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13. ABSTRACT (Maximum 200 Words)
This final report summarized the results obtained from the preparation, evaluation, and extensive SAR analysis of hundreds of compounds with an emphasis on improving the antimicrobial activity of three compound classes outlined in our original proposal. The first class include the substituted pyranosyl cytosines, the second class of compounds are the heterobiaryl guanidines, and the third are bis-heteroarylamidrazones.

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Table of Contents

Cover.....1

SF 298.....2

Introduction.....4

Body.....6

Key Research Accomplishments.....78

Reportable Outcomes.....79

Conclusions.....80

References.....81

Appendices.....83

1 INTRODUCTION

Analysis of the lifestyle of bacteria shows that they have core vulnerabilities that can be exploited by new therapeutic agents. RNA is such a target for drug discovery. Bacterial RNAs perform a variety of core functions, including protein synthesis at the ribosome, processing of tRNAs by RNAse P M1 RNA, and transport of membrane proteins from the cytoplasm by the 4.5S RNA of the signal recognition particle (SRP). Comparative genomics analyses have demonstrated that the core functional elements of these RNAs that have been identified in crystal structures are >98% conserved across all branches of bacterial phylogeny. For the three RNAs listed above, the sequences and/or structures of these targets differ from their eukaryotic counterparts at the core catalytic sites. In addition, each RNA target has pockets that could bind a small drug molecule with high affinity and specificity. These factors make RNA an ideal target for development of effective drugs.

Prior to our award, we had generated three novel classes of small molecules which inhibit bacterial translation at <5 μM *in vitro* using our high-throughput mass spectrometry assay. Our 2002 and 2003 reports outlined the synthetic progress towards optimizing the antibacterial activity of these compound classes. Our current report is a summary of our cumulative efforts to further optimize the continuing classes of compounds, and progress made in animal models of toxicity and infection. Our progress will be discussed within the context of our original proposal.

In our original proposal, the objective of Task 1 was to prepare several hundered pyranosyl cytosines. In this report, the Task 1 section covers the synthetic preparation of these pyranosyl cytosines and the expaned acyclic pyranosyl cytosines. The biological evaluation has been completed and the entire SAR is herein presented. Our original objective for Task 2 was to prepare a vast array of biaryl guanidines and related analogs, and test them in biological assays for antibacterial activity. In Task 2, we summarize the preparation and evaluation of guanidine and, additionally, urea and benzimidazole analogs. The vast number of analogs prepared were possible by using solid-phase and solution phase methodologies. The biological evaluation has been completed and the entire SAR is herein presented; moreover, we include the identification of two urea analogs that were active in antimicrobial assays, and benzimidazoles for futher test in

animal models of infection and toxicity. The original objective of Task 3 was the optimization of bis-arylamidrazones. Task 3, covers the preparation of these carboxamidrazones and their subsequent biological evaluation. The *in vivo* and *in vitro* testing of all compounds was the objective of Task 4 in our original proposal. In this report, Task 4, outlines the *in vivo* animal studies we have performed in which we summarize the protection and toxicity assays in balb-c mice against pathogenic bacteria. It has been convenient for us to report the biological data within sections for Tasks 1-3, and a substantial portion of the work from Task 4 is reported within these sections. We have not yet received data from an extended biowarefare panel, as that work is still pending and we will be pleased to report this in due course. Overall, we have been pleased with our progress and herein present the full results from our 3 years of work in detail.

Additional changes to proposed work plan

Our original proposal (award DAMD17-02-2-0023) was submitted in December of 2000. In the time period between the submission of the proposal, and the work described herein, we have altered our antimicrobial drug discovery strategy. These changes had been outlined and submitted to LTC John Skvorak previously. Herein we highlight those changes again.

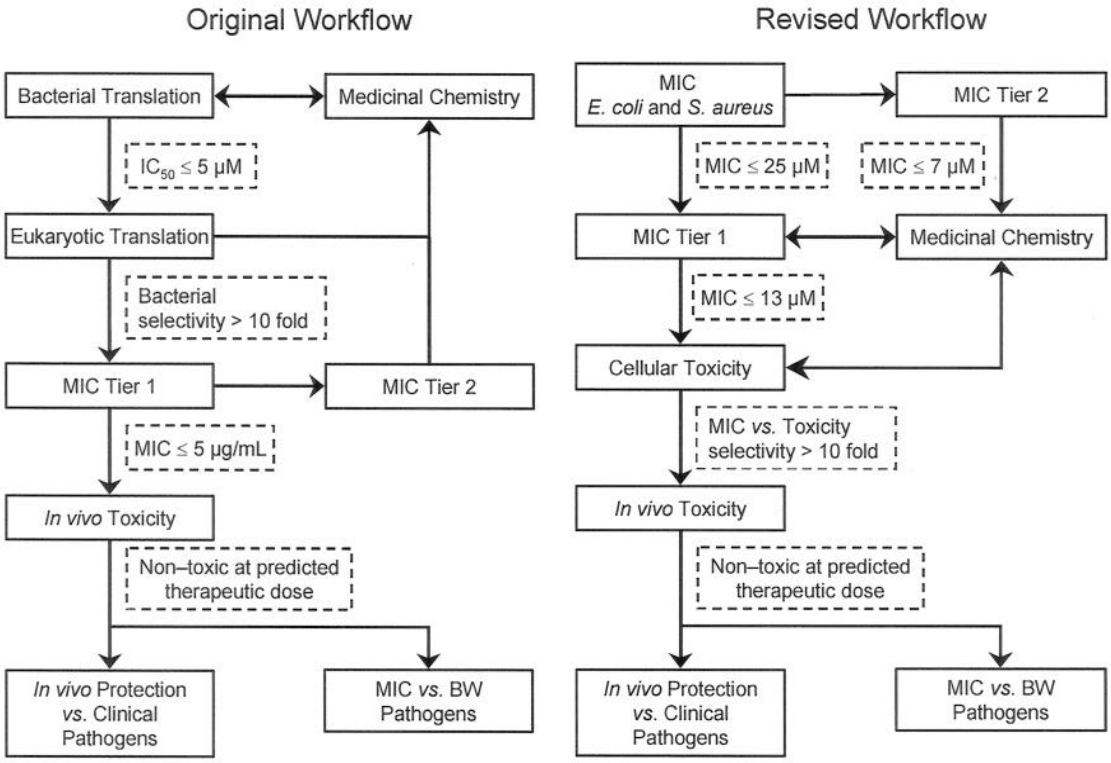
We originally envisioned using *in vitro* translation assays to select which compounds entered antibacterial screening (Figure 1, left panel). This strategy employed both bacterial and eukaryotic translation screens, with a 10x selectivity for bacterial translation inhibition required to advance compounds. Our experience with other classes of antibacterials (not part of this project), combined with our early experience with the pyranosyl cytosine class (task 1) includes the following observations:

- The cell–free translation assays are a simplification of the real process, and have not been as *quantitatively* predictive as we would have liked. They provide a good *qualitative* measure of the mode of action.
- Many compounds which inhibit bacterial translation are not active in MIC type antibacterial assays – this is likely due to poor uptake.
- Many compounds which inhibit eukaryotic translation are not toxic to cells using conventional (MTT) assays, nor are they toxic *in vivo*. This could be due to poor cellular uptake, or differences between the cell–free system and cellular systems, or a combination of both.
- Many compounds exhibit differential uptake by bacterial and eukaryotic cells; and therefore, the cell–free 'therapeutic index' derived from the translation assays often provides guidance which is not relevant to a cellular system.

In general, we have found the cell–free translation assays to be poor predictors of subsequent antimicrobial activity and eukaryotic cellular toxicity. Therefore, we have migrated to a strategy to test compounds in antibacterial MIC assays as quickly as possible (Figure 1, right panel), bypassing the comparative translational screening as soon as compounds having antibacterial activity are identified. We initially screen in two pathogenic tier 1 bacterial strains – *E. coli* (ATCC 25922) and *S. aureus* (ATCC13709). Compounds with MIC < 25 μM in one of the organisms are advanced to the full tier 1

panel. The μM concentration standard is used in place of the more commonly employed

Figure 1. Original (left) and revised (right) project workflow diagrams.



μg/mL standard to better compare molar potencies for medicinal chemistry. Compounds with MIC < 13 μM in one of the tier 1 organisms are counter–screened for human cellular toxicity. For this, we employ the standard MTT method with a human liver cell line. A therapeutic index of 10x is required to move compounds to *in vivo* toxicity screening. Once a compound clears *in vivo* toxicity screening, we then move them towards testing versus BW organisms and *in vivo* protection experiments with our tier 1 pathogenic organisms. These changes do not alter the workload, but save time, as active compounds are moved forward faster. They also focus the medicinal chemistry effort on the optimization of antimicrobial activity, which affords a more responsive optimization cycle, increasing the likelihood of success. As a result of these changes we were able to find two compounds with promising protection against pathogenic bacteria.

2 Research Accomplishments

2.1 Background

Prior to filing our USAMRIID proposal we had worked under DARPA DSO funding to develop a high-throughput mass spectrometry assay that can screen ~10,000 compounds/day against 3 RNA targets. We had correlated these results with RNA-binding and functional assays in the search for molecules that inhibit key RNA functions, and had found the hit rate to be extremely low. For example, we had selected bacterial targets that have either no counterpart or minimal similarity with the equivalent RNA in eukaryotic cells, and have screened >800,000 compounds for activity. The hit rate for finding compounds that bind RNA with <10 μM affinity or disrupt RNA-protein interactions was <0.005%. Despite these low hit rates, we had discovered three classes of molecules that inhibit bacterial translation *in vitro* at levels <10 uM which we believed to be amenable to further chemical modification that would result in the improvement of biological properties. However, none of these compounds were found to be sufficiently potent against pathogenic strains in their current form to warrant preclinical development at that time.

The focus of our USAMRIID proposal was to perform an extensive SAR analysis of these compound classes and to improve their antimicrobial activity. The first class includes the substituted pyranosyl cytosines. These compounds, described in Task 1, have unusual potency (40-400 nM IC_{50}) for inhibiting bacterial translation. A second class of compounds are the heterobiaryl guanidines. These compounds have good activity inhibiting translation (<5 uM), and also show >100-fold specificity for inhibiting bacterial translation. This class is outlined in Task 2. The third class of compounds with good activity in the translation assay are bis-heteroarylamidrazones. Selected members of this class of bis-heteroarylamidrazones previously have demonstrated antifungal and antibacterial activity.(*1, 2*) This data is consistent with a ribosome-associated mechanism, since the fungal rRNA has many similarities with the consensus bacterial sequence at the core catalytic sites. However, none of the compounds significantly (IC_{50}

<100 uM) inhibit bacterial growth in culture. This work is outlined in Task 3. Our *in vitro* evaluation is described in Tasks 1-4 and our *in vivo* work is described in Task 4.

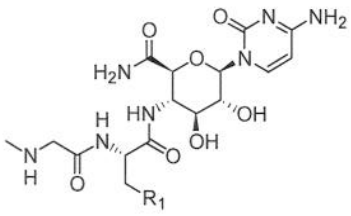
In our 2003 and 2004 annual reports, we described synthetic and screening strategies we implemented to improve the antimicrobial activity and the selectivity for the bacterial ribosome. We also describe the developed solution and solid phase synthesis that allowed us to produce a substantial number of analogs in a rapid and efficient manner; having explored the effects of changes in hydrophobicity, net charge, and functional group placement on the biological activity in prokaryotic and eukaryotic assays. We had additionally reported significant progress in obtaining information which we use to build an SAR dataset for each compound class. Moreover, analyzing each dataset has given us insights into promising modifications that were previously unplanned. Building up on the work we had done, we are currently pleased to furnish this final report, which contains the cumulative progress we have made elucidating the value of these compounds in animal models of protection and toxicity.

2.2 Task 1: Lead Optimization of Pyranosyl Cytosines

In this report, we discuss our progress within the context of our original proposal, in which we synthesized a large number of pyranosyl cytosines analogs related to the naturally occurring gougerotin, bagougeramines, as well as amicetins (Figure 2).(*3, 4*) In general, this class shares a related hexopyranosyl cytosine moiety connected to a modified peptidic side-chain. Similar in both structure and function is the amicetins, which consist of a hexopyranosyl cytosine connected to an aminosugar.(*5*)

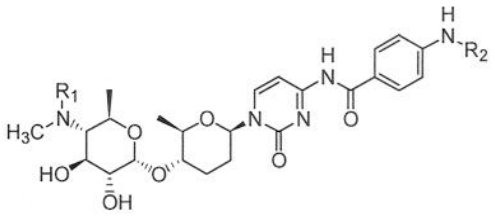
In addition, we report the conclusion of our progress towards determining the structure-activity-relationship (SAR) and understanding of how the activity relates to the MIC required for antibacterial activity.

Figure 2. Two classes of Pyranosyl Cytosines



Gougerotin Class

R_1 = OH, Gougerotin
R_1= NH(CHNH)NH_2, Bagougeramine A



Amicetin Class

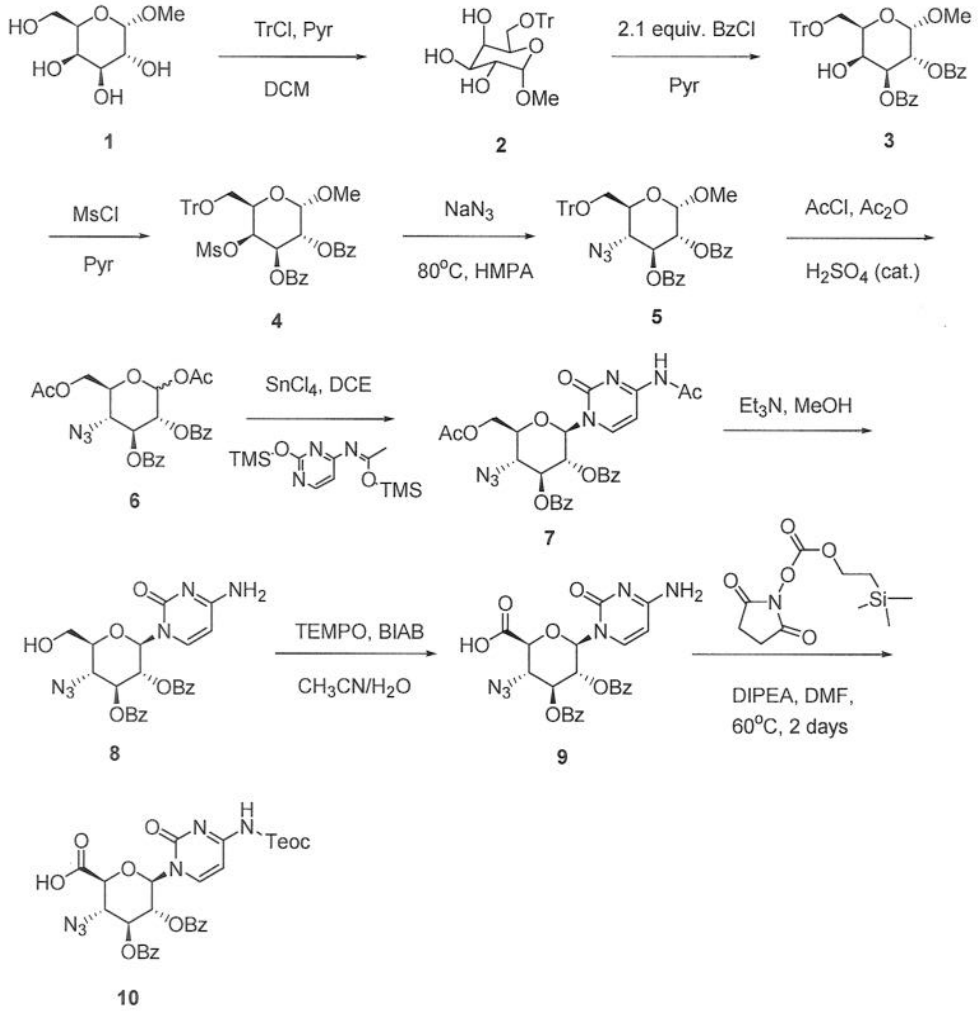
R_1 = H, R_2 = L-Serine, Bamicetin
R_1 = CH_3, R_2 = L-Serine, Amicetin
R_1 = CH_3, R_2 = H, Pliacetin

2.2.1 Synthetic Methods

Synthetically, the most well known of the hexopyranosyl cytosine derived natural products is gougerotin. Gougerotin has been obtained either by isolation from the fermentation broth of *Streptomyces gougeroti(6)* or through total synthesis(*7-14*). No semi-synthetic analogs have been reported in the literature, and no more than thirty analogs(*15*) based on the total synthesis have been reported. Additionally, compounds with improved activity relative to the parent compound, have not been identified, and literature reports of additional synthetic work on gougerotin have remained absent since the 1970s.

Despite gougerotin and its analogs having interesting and potentially useful biological properties as rRNA directed inhibitors of peptide synthesis, the reported synthetic procedures required for their preparation remained lengthy and time-consuming. In fact, several years were required to prepare only tens of compounds. In light of these facts, we sought a more efficient means of generating such analogs, and in particular, we wished to implement recent developments in solid-phase synthesis and combinatorial methodology to greatly accelerate drug discovery based on the gougerotin and amicetin templates. Initially we required an appropriate precursor that could serve as a launching point for a solid-phase synthesis of gougerotin as a test case. This was accomplished as shown in Scheme 1.

Scheme 1. Synthesis of a gougerotin template.



Based on the initial work of Fox and Watanabe,(*14*) we treated α-methyl galactopyranoside **1** with trityl chloride in the presence of pyridine in dichloromethane to effect selective tritylation at the 6-OH group, giving compound **2**. Sugar **2** was then treated with 2.1 equivalents of benzoyl chloride in cold pyridine to benzoylate exclusively at the two free equatorial hydroxy groups to give protected sugar **3**. The remaining axial hydroxy group of compound **3** was functionalized as a mesylate to give compound **4**, which was subsequently treated with sodium azide to effect an Sn2 displacement giving glucopyranose **5**. Treatment of fully functionalized sugar **5** under acetolysis conditions gave our glycosyl donor **6**, which was determined to be identical by

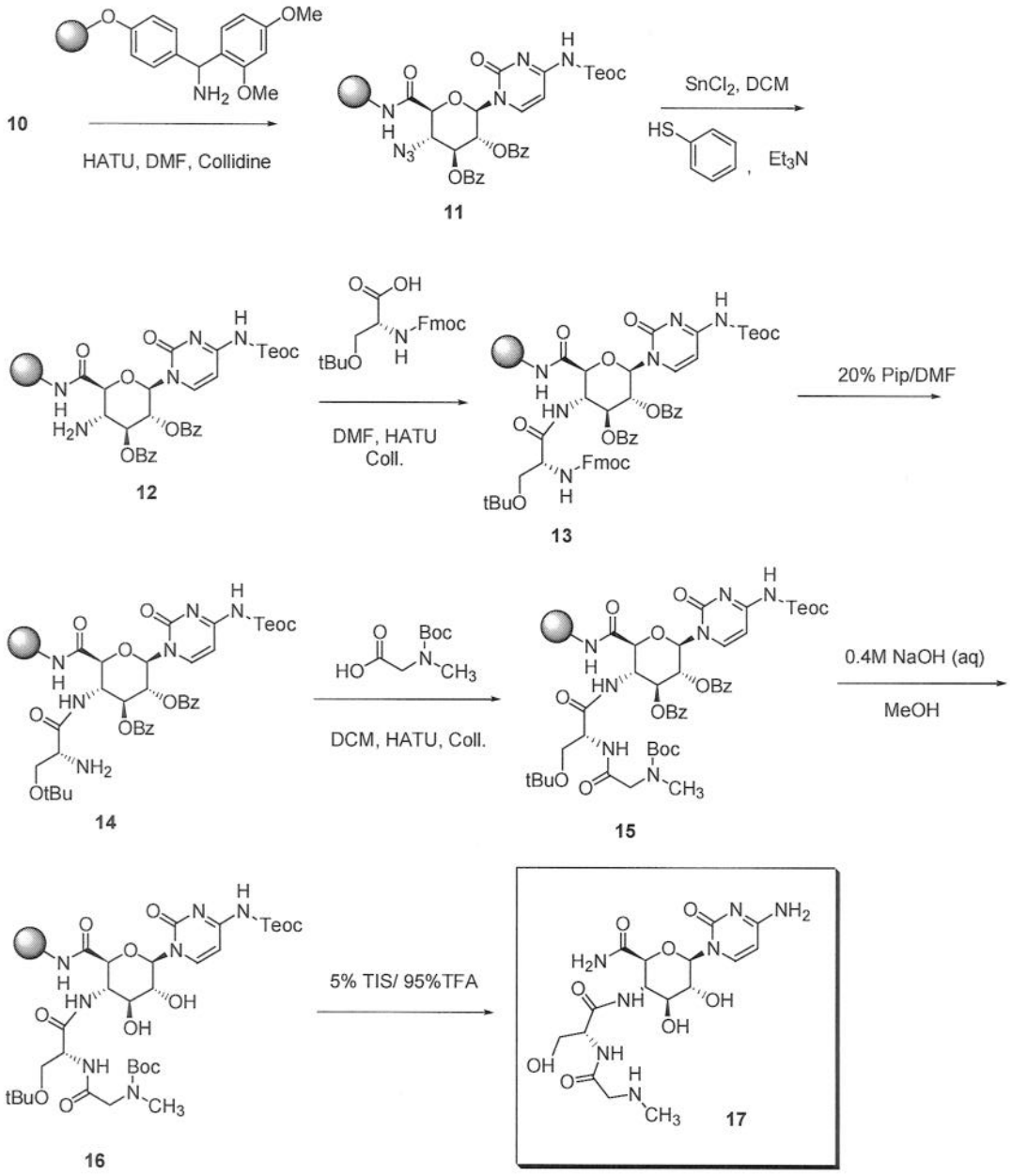
NMR to that previously reported in the literature. Coupling of N-acetyl cytidine with donor **6** under modified Vorbruggen conditions(*16*) gave pyranosyl nucleoside **7**, which was treated with triethylamine in methanol to saponify the remaining acetate protecting groups and afford compound **8**. Oxidation of the free primary hydroxy group of intermediate **8** using TEMPO radical gave carboxylic acid **9**.(*17*) Finally, protection of the exocyclic N-4 of compound **9** as a trimethylsilylethylcarbamate (Teoc)(*18*) gave the fully protected precursor **10**.

To demonstrate the likelihood of success of adapting the gougerotin template for use in solid-phase combinatorial chemistry we prepared the parent, gougerotin, using solid-phase methodology. As shown in Scheme 2, precursor **10** was coupled to ArgoGel® rink resin using HATU in DMF to give the resin bound amide **11**. Resin **11** was then treated with tin(II) to effect a complete reduction of the azide to give amine **12**. Functionalizing the free amine of compound **12** with a protected D-serine gave resin **13**. Removal of the Fmoc protecting group of resin **13** gave resin **14**, which was immediately reacted with N-Boc-sarcosine to give the advanced intermediate **15**. Removal of the benzoyl protecting groups of resin **15** to give resin **16**, followed by deprotection with TIS/TFA gave gougerotin (**17**) which was characterized by NMR and Mass Spec. and determined to be >90% pure by HPLC (ELSD(*19*) detection), thus demonstrating the success of our methodology.

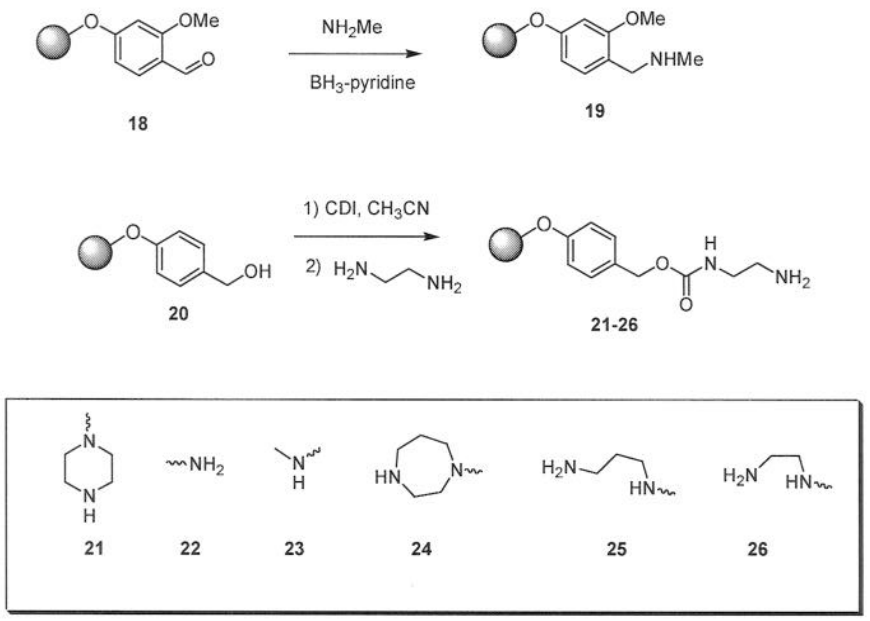
2.2.2 Modification of 6'-Carboxyl Group

Having demonstrated an efficient solid-phase method for the preparation of gougerotin, we wished to extend our procedure to encompass additional diversity elements at the 6'-carboxyl group. In particular we were interested in preparing functionalized resins that could be used to prepare precursors that would be used in a divergent fashion. Therefore, two separate methods were used to obtain resins.

Scheme 2. The solid-phase total synthesis of gougerotin.



Scheme 3. The Synthesis of 6'-Functionalized Gougerotin Analogs.



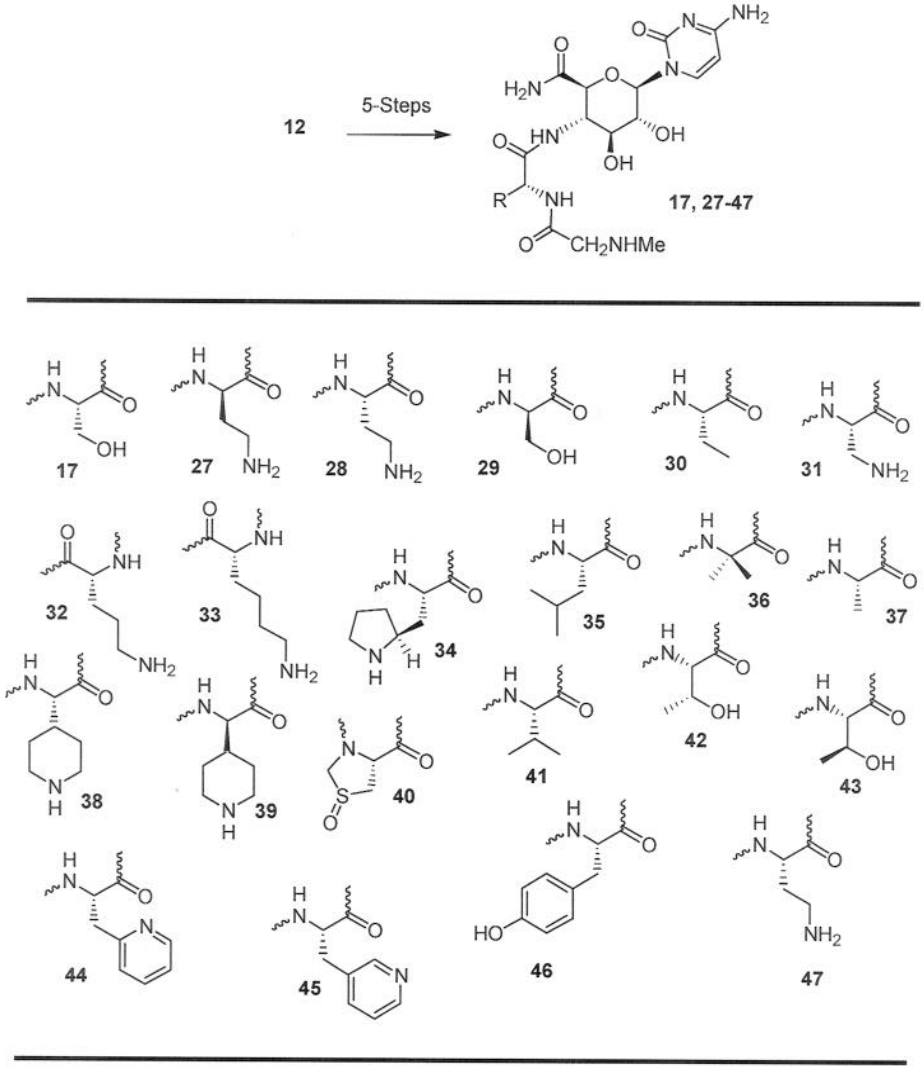
First, ArgoGel-MB-CHO resin **18** was treated with methylamine in the presence of BH_3-pyridine to affect a reductive amination.(*20*) Second, in the case where a diamine was used, ArgoGel-Wang resin was first treated with carbonyldiimidazole (CDI) and then reacted with the diamine to give functionalized resins **21-26**.(*21*) In this way, one amine could be selectively protected with the cleavable carbamate linker, while preserving the other amine for functionalization with the gougerotin precursor. Each amine was then coupled to the appropriate precursor and treated as in Schemes 1 and 2.

2.2.3 Modification of the N-4' Side Chain.

The first and most straightforward modifications that were prepared at the N-4' side chain involved direct substitution of one or more of the amino acids shown in Scheme 2 with an alternative amino acid or carboxylic acid. The modifications made at the first residue are shown in Scheme 4. A wide range of substituents were chosen including cyclic (**34, 38-40**), acyclic (**27-33, 35-37, 41-43**), heterocyclic aromatic (**44-45**) and heterocyclic non-aromatic (**34, 38-39**). Additionally, the lipophilicity, position of the charge, hydrophilicity, sterics and chiral center were all varied. Moreover, these

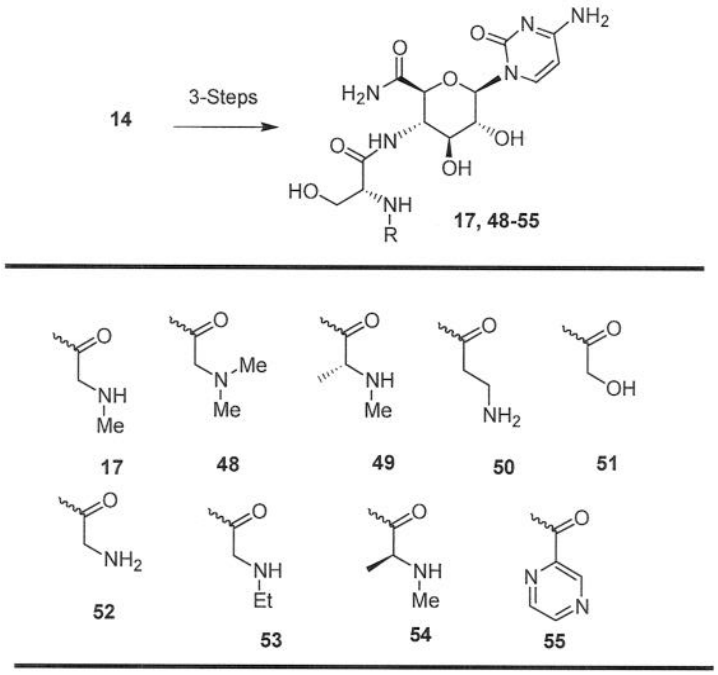
modifications were made in conjunction with modifications at the other positions so as to result in a library of several hindered analogs.

Scheme 4. Examples of N-4' Functionalization at the First Residue.



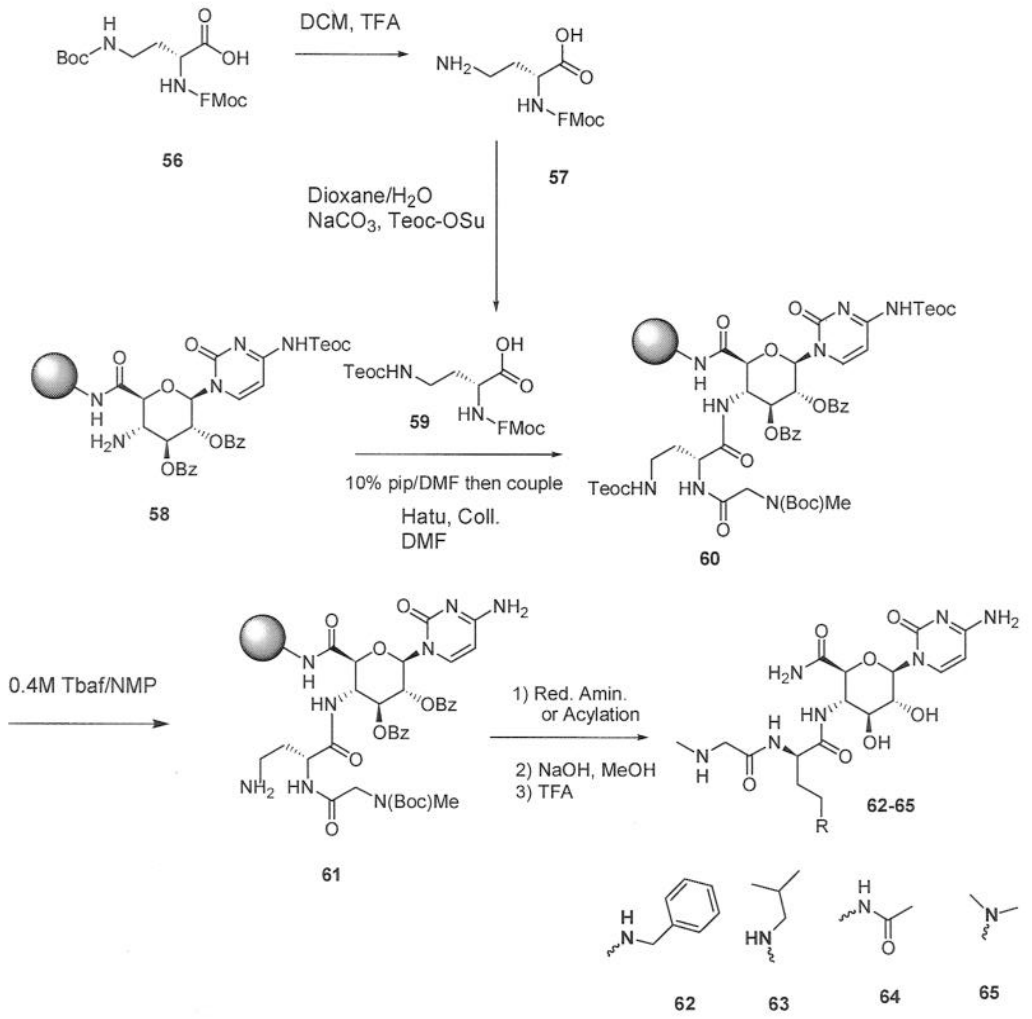
Next, we varied the residue at the second position. Initially, we found that only slight variations to structure were tolerated (*Vida infra*), and so we focused only on slight deviations from the naturally occurring sarcosine residue. Scheme 5 illustrates the modifications that were made and tested (**48-55**).

Scheme 5. N-4' Functionalization at the Second Residue.



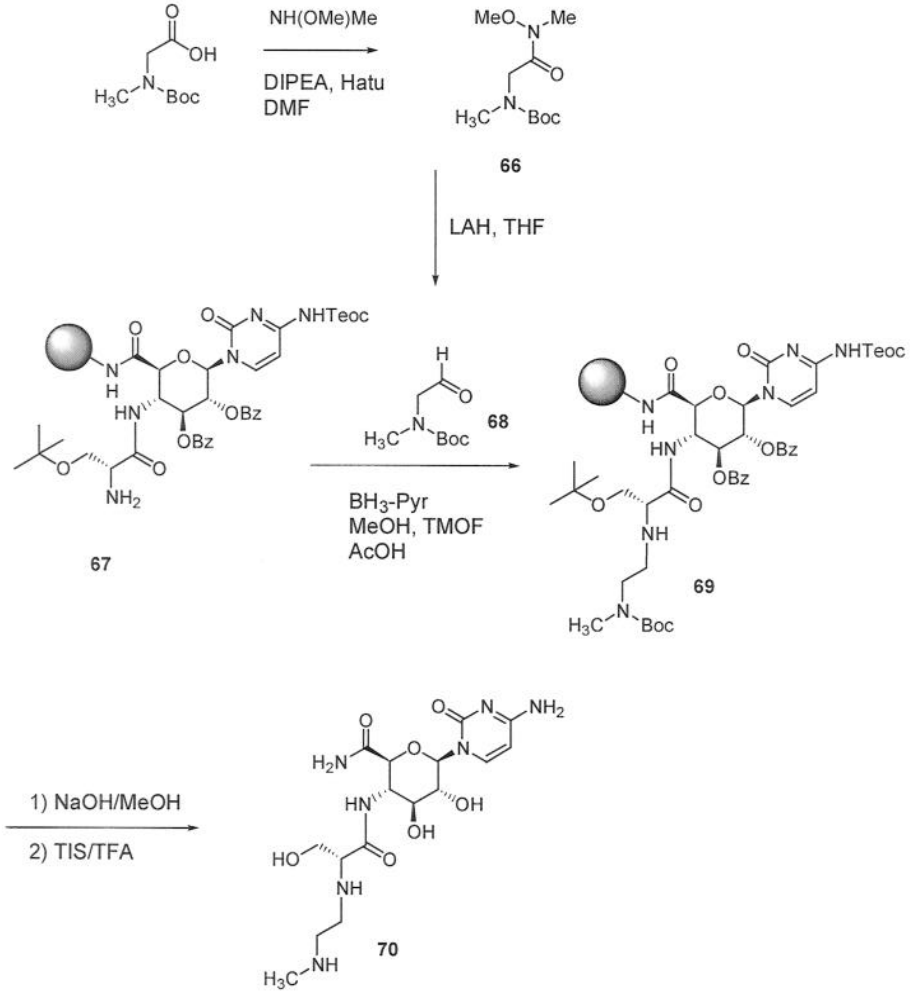
Having established a rapid and efficient synthesis of N-4' side chain analogs using alternative amino acids and carboxylic acids, our attention turned to the synthesis of further modification of the first residue in a more convergent manner. Specifically, we wished to introduce a N-protected side chain that could be removed during our synthesis, and before cleavage from the resin, which would result in release of a free amine. Reductive amination and alkylations could then be carried out and we could further explore the space about this important side chain. Therefore, the commercially available diaminobutyric acid (DAB) derivative **56** was treated with trifluoroacetic acid and then Teoc-NHS(*18*) to give **59** containing a fluoride-labile protecting group (Scheme 6). This could then be substituted into our general reaction scheme to give the Teoc-protected precursor **60** from **58** using previously described methodology. This precursor was then treated with TBAF in NMP to liberate the free amine (i.e. Compound **61**). Several reductive amination were then carried out (benzaldehyde (**62**), formaldehyde (**65**) and isobutyraldehyde (**53**) in addition to acylation (**63**).

Scheme 6. Synthesis using a Modified DAB residue.



Lastly, we required access into N-4' analogs that would be stable to peptidic cleavage, and chose to prepare analogs that were reduced at the second residue (i.e. replacement of the carbonyl with CH_2). Therefore, the Weinreb(*22*) amide of sarcosine (**66**) was prepared and then treated with lithium aluminum hydride (LAH) to affect a selective reduction to aldehyde (**68**). This was then reacted with precursor **67**, under our standard reductive amination conditions to give intermediate **69**. Carrying out the rest of our procedure as previously demonstrated gave us analog **70**. This was repeated using different substituents at the 6'-carboxy terminus as well as with two different N-4' side chains at the first residue.

Scheme 7. Synthesis of Reduced Sarcosine Analogs.



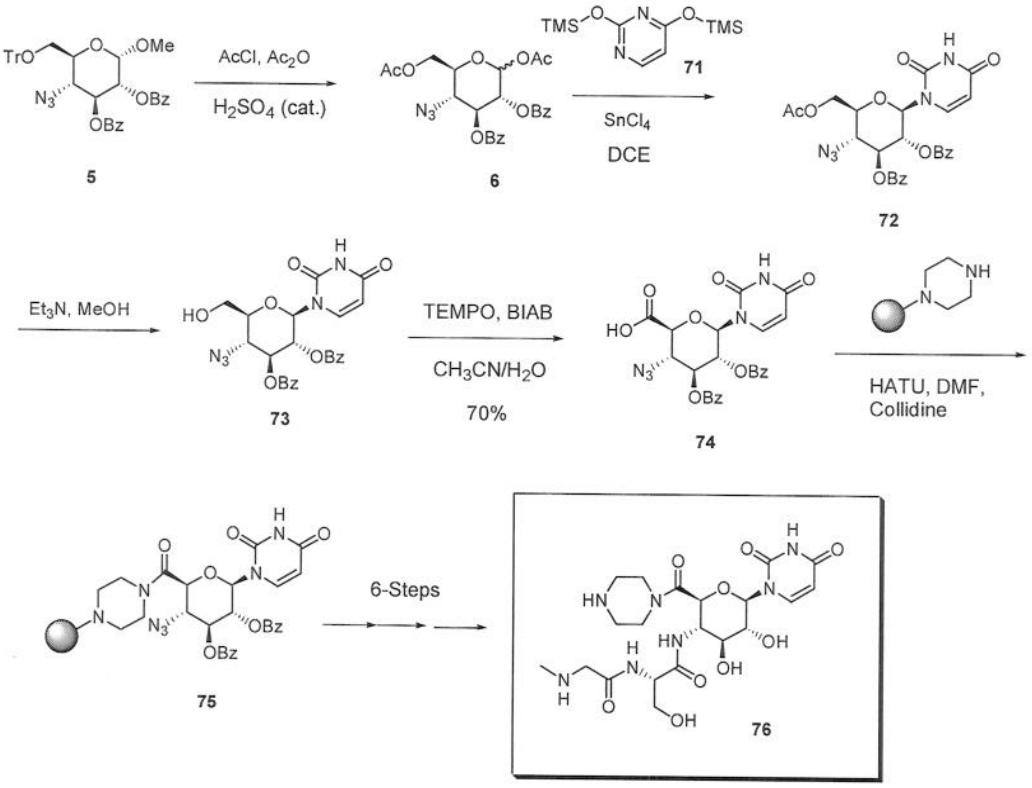
2.2.4 Modification of the C-5 and C-6 Positions.

Preparation of the C-5 and C-6 positions were important for several reasons. First, modification may prove to be crucial in averting any cellular deaminases, a well known resistance mechanism for Blasticidin S.(*23*) Second, amicetin as well as other pyranosyl cytosines have N-substituents which may prove important in binding and avoiding cellular enzymes which deactivate these antibiotics. Lastly, these positions constitute an important branching point for achieving additional structural diversity.

Modification at the C-5 position seemed to be less straight forward than modification of the C-6 position. We had hoped that we could modify a procedure,(*24*) in which a uracil derivative was transformed to a C-6 triazole derivate which could then

be displaced with a nucleophile. However, no reports of this reaction on the solid-phase could be found in the literature. Furthermore, due to the highly heterogeneous nature of the triazolating reagent it was unclear if this reaction would work on the solid-phase. In order to test our hypothesis we first prepared the uracil precursor **75** (Scheme 8).

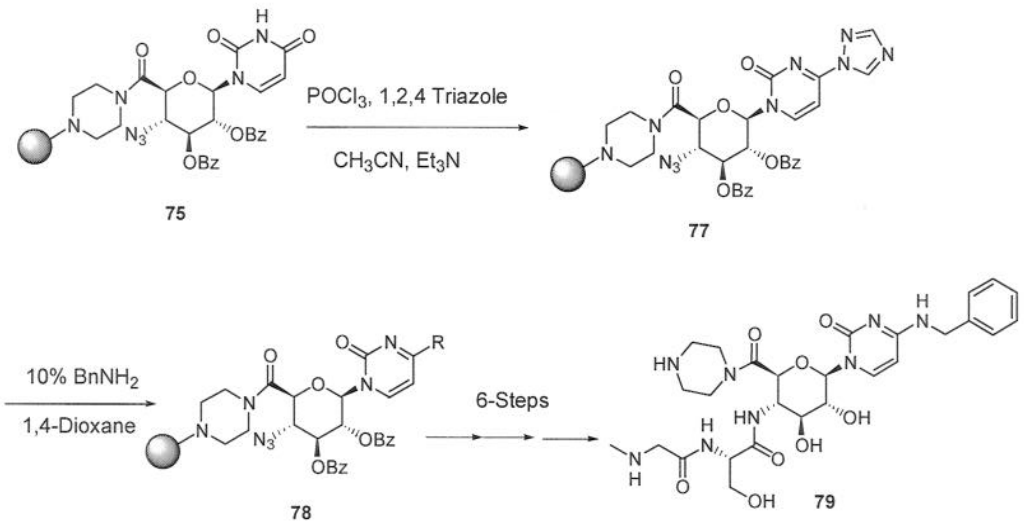
Scheme 8. Synthesis of C-6 Uracil Analogs.



Coupling silylated uracil (**71**) to our glycosyl donor **6** under modified Vorbruggen conditions(*16*) using $SnCl_4$ as the Lewis acid to give a very small yield of product **72**. Subsequently it was found that using 3 equivalents of TMS-OTf under refluxing conditions greatly improved our yield to over 80%. After deacteylation and oxidation using the conditions developed for the protected cytidine, we were able to obtain our precursor **74**. A HATU mediated coupling then gave our resin-bound uracil derivative **75**. Finishing out our synthesis using the protocol illustrated in Scheme 2 gave our Uracil

derivative **76**. Additional derivatives, functionalized at C-6' and N-4' were also prepared (see details in biological evaluation section 2.2.5).

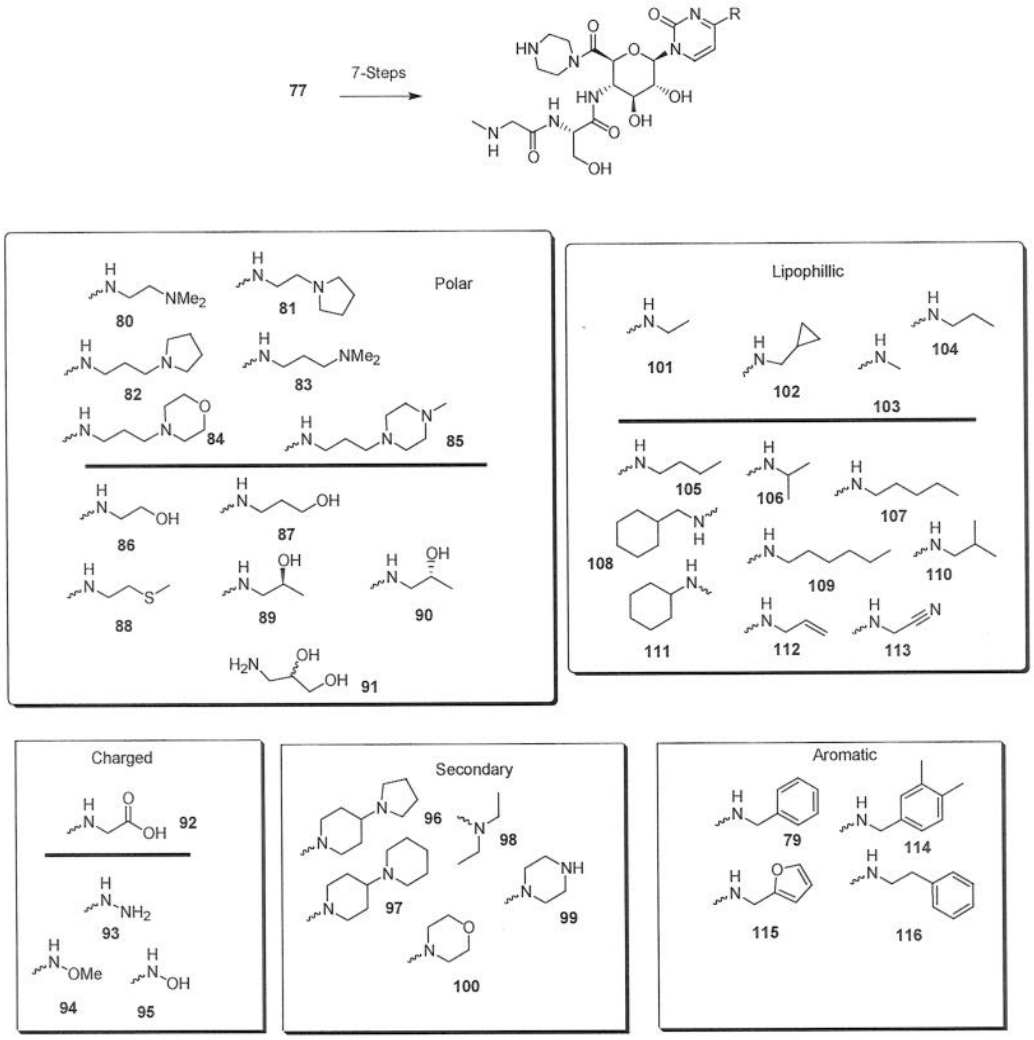
Scheme 9. Conversion of Uracil Derivatives to Cytidine Derivatives.



With our uracil derivative in hand, we treated the intermediate **75** with the preformed triazolating reagent (i.e. 1,2,4-triazole, $POCl_3$ and Et_3N in CH_3CN) for 4 hours followed by nucleophilic aromatic substitution with benzylamine in ethanol or dioxane gave the putative intermediate **78**. A small amount of this resin was then deprotected with TFA and tested by LCMS ([M+H]= 610.2 *m/z*) to indicate that the reaction was indeed successful. The remainder of the sequence was affected (coupling both fragments and deprotection) to give the N-4 substituted gougerotin precursors **79**. The analogous procedure was then carried out to give a large array of C-6 substituted analogs for biological testing (Scheme 10). Different types of substituents were chosen so as to fully explore the parameters set out in our original proposal; e.g. Polarity , charge, steric space and lipophilicity.

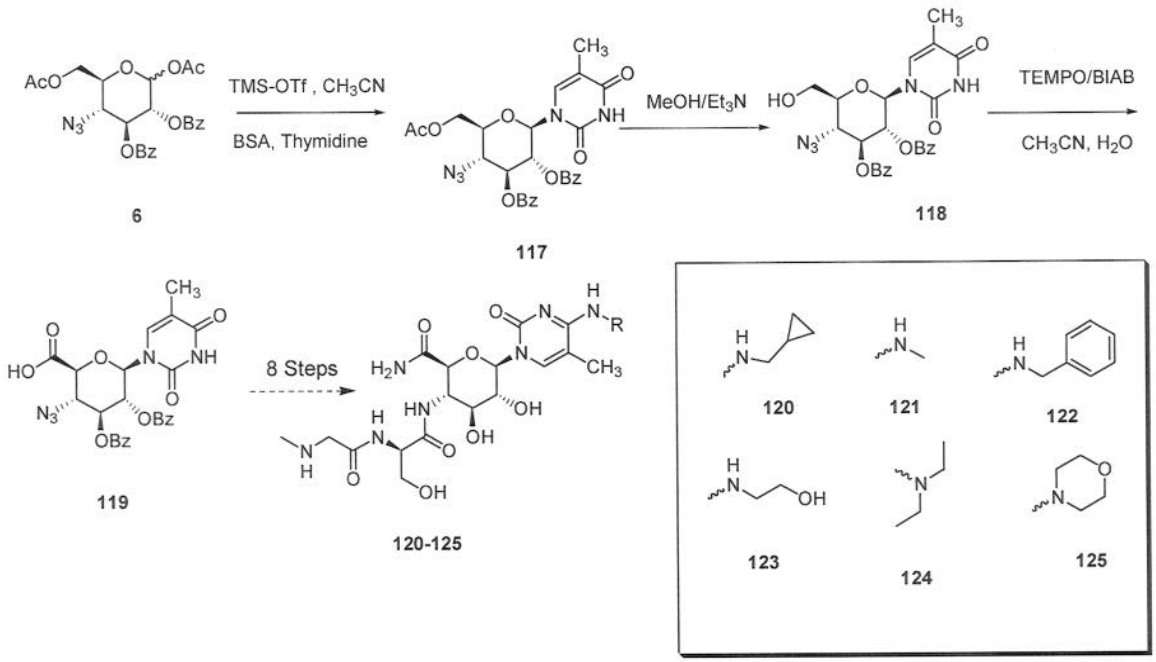
With the synthesis of the C-6 Substituted analogs worked out, the synthesis of our C-5 substituted analogs could now be prepared by taking advantage of our triazolation-displacement chemistry. Therefore, we treated 5-methyl uracil under conditions analogous to Scheme 8, and prepared several C-5 methyl analogs (**120-125**) as shown in Scheme 11.

Scheme 10. Several C-6 Analogs Prepared.



In addition to compounds **120-125,** we also prepared the corresponding analogs in the rink piperazine series. Biological evaluation of all of the compounds is reported in section 2.2.5.

Scheme 11. C-5 Methyl Analogs Prepared.



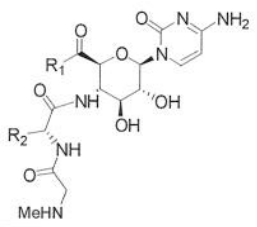
2.2.5 Biological Evaluation of the Gougerotins.

The biological evaluation of C-6' carboxyl modified analogs is shown in Table 1 and 2. As shown, biological testing for transcription/translation activity, as well as for the minimum inhibitory concentration against a pathogenic strain of *E. Coli*, was carried out. This initial biological screening was very encouraging, and the activity of several of our analogs, i.e. **127** and **128**, showed markedly improved activity over the parent compound, gougerotin, against pathogenic *E. Coli* (2-5 fold improvement). (Testing against *S. Aureus* was also carried out against all compounds herein, with no significant activity observed when the organism is not indicated in the table). In particular, the piperidine analog imparted an increase in MIC activity without a significant change in transcription/translation. Moreover, over half of our analogs were excellent inhibitors of translation, with IC_{50} values better or equal to the natural product. Interestingly, a single methyl substitution appeared to be the least favorable modification (**135-139**) and is surprising considering the small extent to which it varies with the parent compound as compared the other analogs. On the other hand, modifications such as homopiperazine

seemed to behave similar compared to the parent piperazine modification.

Likewise, the ring-opened analogs of piperazine (i.e. **145-154**) were similar in profile. Many different modifications seemed to be well tolerated at the 6'-position, and an increase in activity could be realized in the piperazine series.

Table 1. Biological Activity of 6'-Carboxy Modified Analogs.



Compound	R_1	R_2	Translation IC_{50} (μM)	MIC (μM) ATCC 25922
126	a	g	0.5	>100
127	a	h	0.3	75-50
128	a	i	0.2	100
129	a	j	3.2	>100
130	a	k	.03	150-100
17	b	g	0.5	>200
131	b	h	0.03	>100
132	b	i	.02	>100
133	b	j	1.0	>100
134	b	k	0.02	>100
135	c	g	1-10	>100
136	c	h	0.03	>100
137	c	i	0.3	>100
138	c	j	1-10	>100
139	c	k	0.4	>100

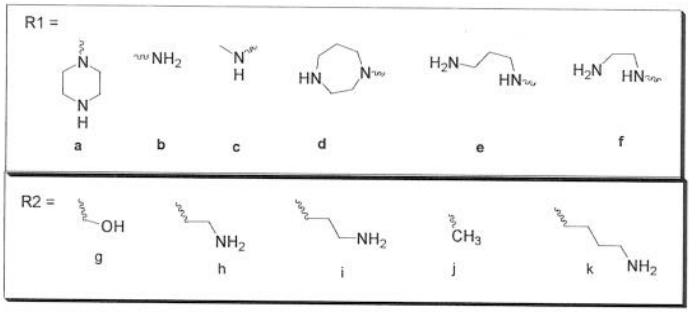
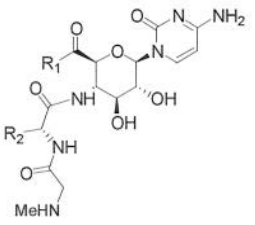
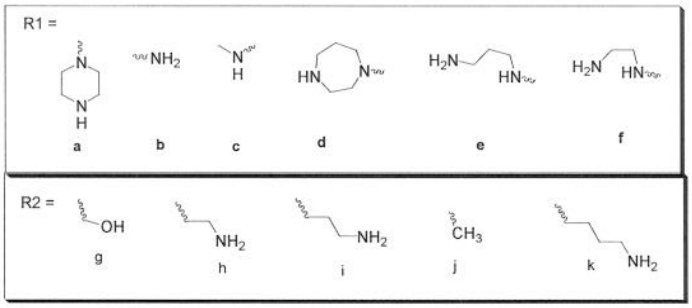


Table 2. Biological Activity of 6'-Carboxy Modified Analogs (Continued).



Compound	R_1	R_2	Translation IC_{50} (μM)	MIC (μM) ATCC 25922
140	d	g	0.8	>100
141	d	h	0.7	>100
142	d	i	0.5	>100
143	d	j	1-10	>100
144	d	k	0.7	>100
145	e	g	0.6	>100
146	e	h	0.4	>100
147	e	i	0.6	>100
148	e	j	0.5	>100
149	e	k	1-10	>100
150	f	g	0.5	>100
151	f	h	0.4	>100
152	f	i	0.4	>100
153	f	j	.04	>100
154	f	k	1-10	>100

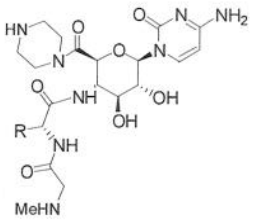


Tables 1 and 2 also demonstrate our first look at the SAR around the first peptidic residue of the side chain. Immediately it is apparent the diaminobutyric acid analog imparts an increase in activity, when used in conjunction with the piperazine amide at the

6'-position. In particular, compound **127** proved to be our best compound early on in this series. In addition the modifications demonstrated in tables 1 and 2, several other modifications at the first peptidic residue of the N-4' side chain are shown in Table 3 and 4.

Table 3. Biological Activity of Modifications at the First Residue of N-4' Side Chain.

a-series



b-series

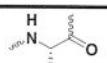
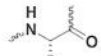
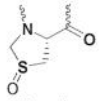
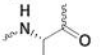
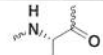
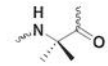
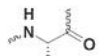
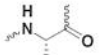
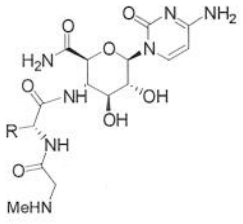
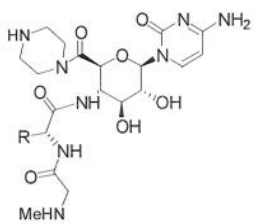
Compound	Series	R	Translation IC_{50} (μM)	MIC (μM) ATCC 25922
30	a		10-100	>100
155	b		>100	>100
31	a		0.2	>100
156	b		3.2	>100
40	a		6	>100
157	b		10-100	>100
42	a		10-100	>100
158	b		10-100	>100
43	a		>100	>100
159	b		10-100	>100
36	a		>100	>100
160	b		10-100	>100
35	a		>100	>100
161	b		>100	>100
41	a		>100	>100
162	b		10-100	>100

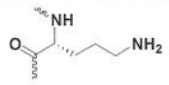
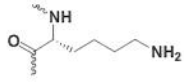
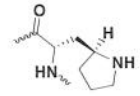
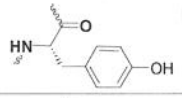
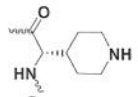
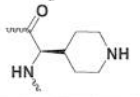
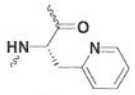
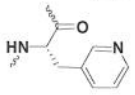
Table 4. Biological Activity of Modifications at the N-4' Side Chain (Continued).



a-series

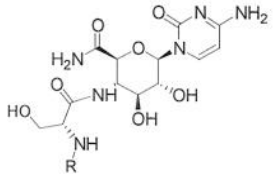


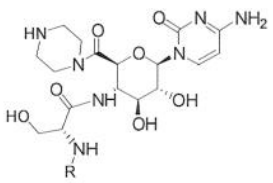
b-series

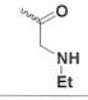
Compound	Series	R	Translation IC_{50} (μM)	MIC (μM) ATCC 25922
132	a		.02	100
128	b		0.2	>100
134	a		.03	>100
130	b		.02	150-100
34	a		0.2	>100
163	b		0.2	>100
164	a		10-100	>100
165	b		10-100	>100
38	a		10-100	>100
166	b		10-100	>100
39	a		>100	>100
167	b		10-100	>100
44	a		10-100	>100
168	b		10-100	>100
45	a		10-100	>100
169	b		10-100	>100

The most apparent trend was the decrease in activity (T/T, MIC) when the terminal amino group or hydroxy group is replaced with its hydrocarbon congener. This may indicate that there is an important hydrogen bond acceptor nearby which serves as a key interaction. Moreover, it is apparent that an increase in branching at this residue is also unfavorable. This seems to point to a pharmacophore in this particular area having

Table 5. Biological Activity of Second Residue of N-4' Side Chain.



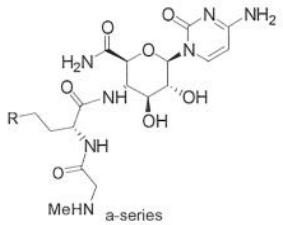


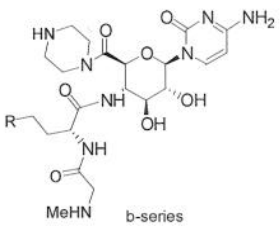
	a-series			b-series
Compound	Series	R	Translation IC_{50} (μM)	MIC (μM) ATCC 25922
170	a	**H**	>100	100
171	b	**H**	>100	>100
52	a		4	>100
130	b		2	100
48	a		10-100	>100
172	b		10-100	>100
53	a		2	>100
173	b		5	>100
49	a		10-100	>100
174	b		10-100	>100
54	a		10-100	>100
175	b		2	>100
49	a		>100	>100
176	b		10-100	>100
54	a		>100	>100
177	b		>100	>100

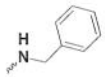
some fairly stringent steric constraints. For example, compounds 158-159 are 20-200 fold less active then the unbranched congener, while compounds 130, 132 and 134 were 20 fold more active than gougerotin. These constraints are also quite apparent in the second peptidic residue also. As shown in Table 5, branching alpha to the carbonyl leads to a decrease in activity, however, in this case the eudismic ratio is slightly better indicating a pharmacophore model in which there is less steric constraint on one side of the binding pocket.

Moreover, it is important to have at least on proton available on the terminal nitrogen, as a dimethyl (48, 172) has reduced activity, while either the analog with two protons (52, 130) or containing an ethyl group (53, 173) is quite comparable to the naturally occurring sarcosine. Extending the chain (49, 176) had a negative effect in the T/T assay, indicating that a one carbon spacer (between the carbonyl and nitrogen) is probably ideal.

Table 6. Biological Activity of Modified DAB Analogs.





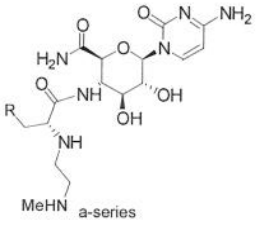
Compound	Series	R	Translation IC_{50} (μM)	MIC (μM) ATCC 25922
63	a		1-10	100
178	b		1-10	>100
65	a		0.5	>100
179	b		0.4	100
62	a		10-100	>100
180	b		0.6	>100
64	a		1-10	>100
181	b		1.3	>100

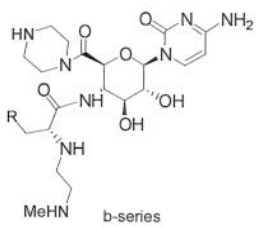
The modified DAB analogs are shown in Table 6. It is clear that there is much less steric constraint at this position, as compared with the terminal residue. In fact, even the dimethyl analogs (65, 179) were active in contrast to the dimethyl in Table 5 (48, 172).

The reduced analogs are shown in Table 7. It was demonstrated in Table 5 (compounds **170** and **171**) that if the sarcosine residue is not present, the resultant compound is completely inactive. A bacteria with such a protease could deactivate gougerotin, and we postulated that this could be one of the modes that bacteria have evolved to protect itself against the pyranosyl cytosines. In order to test our hypothesis,

we prepared analogs **70, 182-184** missing the carbonyl oxygen adjacent to the first sarcosine nitrogen. As compound **182** illustrates, we still retain excellent T/T activity without a corresponding increase in MIC, consistent with deactivation by a protease as not being a major mode of deactivation. Additionally, it seems reasonable that the carbonyl may even serve the positive role of orienting the terminal sarcosine nitrogen in the right position, as compounds **70**, **182** and **184** were less active than their parents, compounds, **17, 31** and **126**, respectively.

Table 7. Biological Activity of Reduced Analogs.

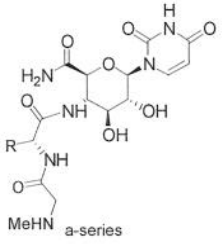




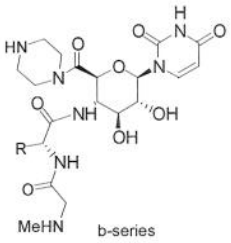
Compound	Series	R	Translation IC_{50} (μM)	MIC (μM) ATCC 25922
70	a	**OH**	1-10	>100
182	b	**CH_2NH_2**	0.5	>100
183	a	**CH_2NH_2**	10-100	>100
184	b	**OH**	10-100	>100

Another mode of deactivation that has been well established in the Blasticidin S series, is by the use of a very selective and facile deaminase (in the case of Blasticidin it is referred to as BSD or blasticidin deaminase.) These ubiquitous enzymes deaminate the cytidine base of Blasticidin to yield the uracil congener, which is completely inactive. We had postulated that a similar mode of action for the gougerotin class may be responsible for the lack of antibacterial activity in the pathogenic strains of *E. Coli* that we were testing in. To test this hypothesis, we generated a series of uracil derivatives (Table 8) and tested then in our T/T assay and ascertained the MIC. Consistent with our hypothesis, these analogs were >20 fold less active than their cytidine parents (although no gougerotin deaminase has been identified as of this report).

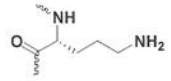
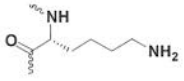
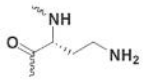
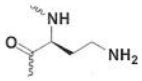
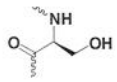
Table 8. Biological Activity of Uracil Analogs.



a-series

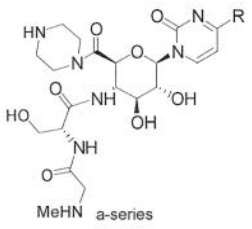


b-series

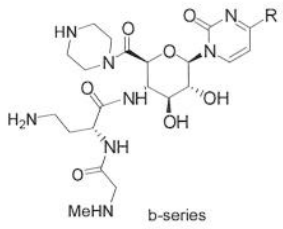
Compound	Series	R	Translation IC_{50} (μM)	MIC (μM) ATCC 25922
185	a		>10	>100
186	b		>10	>100
187	a		>10	>100
188	b		>10	>100
189	a		>10	>100
76	b		>10	>100
190	a		>10	>100
191	b		1-10	>100
192	a		>10	>100
193	b		>10	>100
194	a		>10	>100
195	b		0.5	>100

To test our hypothesis we wished to make a series of N-6 substituted analogs. Such substitutions on cytidine itself have been well established to be inhibitors of cytidine deaminase, and nature most likely would have retained similar constraints in the any putative gougerotin deaminase. Moreover, we wished to access the N-6 substituted analogs as the amicetins and other biologically modified Blasticidins (among other pyranosyl cytidine) are appended at this position with a substituent; the most common substituent being a polar head group (such as an amino acid residue or derivative.)

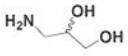
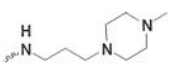
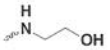
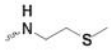
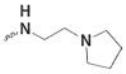
Table 9. Biological Activity of C-6 Polar Analogs.



a-series



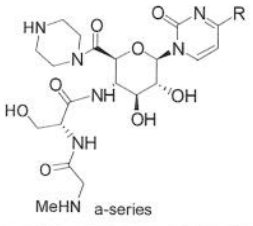
b-series

Compound	Series	R	Translation IC_{50} (μM)	MIC (μM) ATCC 25922
87	a		10-100	100
196	b		1.5	>100
89	a		10-100	>100
197	b		10-100	100
90	a		10-100	>100
198	b		0.9	>100
91	a		10-100	>100
199	b		2.1	>100
85	a		0.9	>100
200	b		0.5	50-100
86	a		10-100	>100
201	b		0.9	>100
88	a		1.2	>100
202	b		18	>100
86	a		10-100	>100
203	b		0.6	50-100

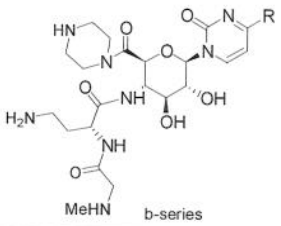
There fore we prepared a vast array of N-6 substituted analogs encompassing polar derivatives (Tables 9 and 10), secondary amines, (Table 11), lipophilic groups (Tables 12 and 14) and aromatic groups (Table 13). In the polar series, it was interesting to note the large number of analogs that were well tolerated at this position. Specific trends include; the head groups with amino groups tended to be better than the hydroxy groups, the piperazine series was more tolerant of substituents than the serine series, steric bulk seemed to play little role in excluding active analogs and hydrogen bond donors were tolerated in addition to acceptors. Substitution with secondary amines are shown in Table

11. Compounds **98** and **214** were clearly active indicating that even secondary amines are well tolerated at this position, however, it is also clear that secondary amines are not as good as primary.

Table 10. Biological Activity of C-6 Cytidine Polar (Continued).



a-series



b-series

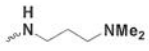
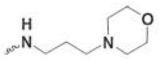
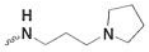
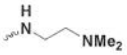
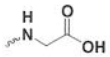
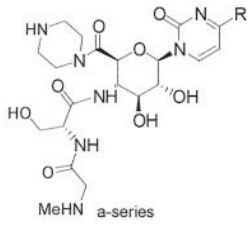
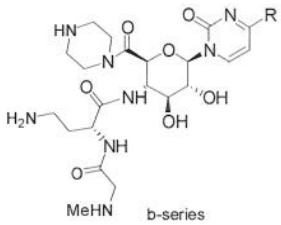
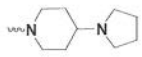
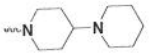
Compound	Series	R	Translation IC_{50} (μM)	MIC (μM) ATCC 25922
83	a		0.6	>100
204	b		0.2	50-100
84	a		1.6	>100
205	b		0.6	50-100
82	a		1.2	>100
206	b		0.4	50-100
80	a		10-100	>100
207	b		0.5	50-100
92	a		10-100	>100
208	b		0.4	>100
95	a		1.3	>100
209	b		0.9	>100
93	a		10-100	>100
210	b		10-100	>100
94	a		10-100	>100
211	b		10-100	>100

Table 11. Biological Activity of C-6 Cytidine Secondary Analogs.

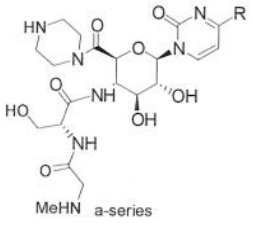




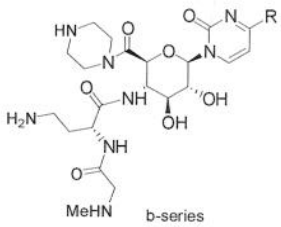
Compound	Series	R	Translation IC_{50} (μM)	MIC (μM) ATCC 25922
96	a		>100	>100
212	b		1.0	50-100
97	a		10-100	>100
213	b		10-100	50-100
98	a		2.2	>100
214	b		1.2	50-100
100	a		10-100	>100
215	b		10-100	50-100
99	a		>100	>100
216	b		10-100	>100

Shown in Table 12, a homologous series of lipophilic alkyl groups were prepared and tested for activity. A slight trend is readily observed in that increasing the hydrocarbon chain length to propyl or butyl seems to be optimal. Shorter or longer chains result in less active compounds, although this trend is much more pronounced in the serine series over the dab series. Interestingly, the dab-substituted analogs containing the lipophilic chains were demonstrated to have antibacterial activity equal to the unsubstituted analog. Additionally, the increased steric bulk of compounds **216** and **217**, seem to have a very minimal effect attenuating activity. This is again consistent with the data we have obtained in the polar series of compounds. In fact, in some cases it has proven to be favorable to have a lipophilic substituent over a polar one (compare **87, 105**).

Table 12. Biological Activity of C-6 Cytidine Lipophilic Analogs.



a-series



b-series

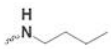
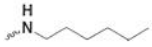
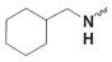
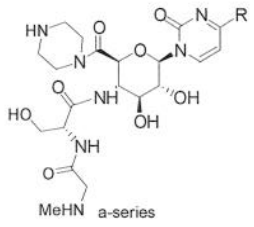
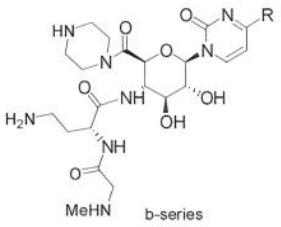
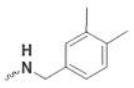
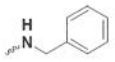
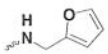
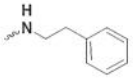
Compound	Series	R	Translation IC_{50} (μM)	MIC (μM) ATCC 25922
103	a		10-100	>100
217	b		0.9	>100
101	a		0.5	>100
218	b		0.5	50-100
104	a		1.4	>100
219	b		0.4	50-100
105	a		1.3	>100
220	b		1.1	>100
107	a		4.5	>100
221	b		1.4	50-100
109	a		10-100	>100
222	b		3.4	>100
102	a		1.3	>100
223	b		0.3	50-100
106	a		10-100	>100
224	b		2.0	>100
108	a		10-100	>100
225	b		10-100	>100
111	a		10-100	>100
226	b		4.6	50-100
112	a		10-100	>100
227	b		10-100	>100
113	a		10-100	>100
228	b		1.5	50-100

Table 13. Biological Activity of C-6 Cytidine Aromatic Analogs.



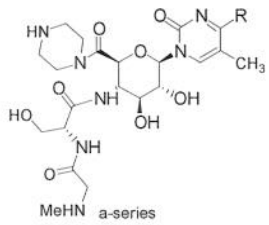


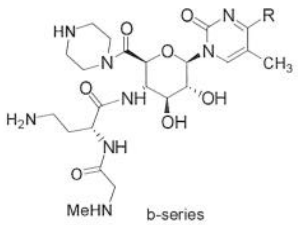
Compound	Series	R	Translation IC_{50} (μM)	MIC (μM) ATCC 25922
114	a		0.7	>100
229	b		0.6	25-50
79	a		10-100	>100
230	b		0.4	50-100
115	a		0.6	>100
231	b		0.4	50-100
116	a		2.3	>100
232	b		0.5	25-50

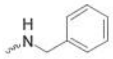
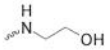
Among the most promising C-6 substituted analogs are the aromatic C-6 head groups shown in Table 13. In particular, the phenethyl analog **232** and substituted benzyl analog **229** were 8-16 fold more active than gougerotin. This was among the most recent analogs that we have prepared and still have not explored the countless other aromatic groups that are available.

Lastly, we prepared a series of C-5, C-6 disubstituted analogs. Unfortunately, these analogs were completely inactive. This may be due to a strict steric constraint of the pharmacophore on that side of the heterocycle, or due to the 5-methyl group orienting the hydrogen away from its putative acceptor.

Table 14. Biological Activity of C-5 Analogs.





Compound	Series	R	MIC (μM) Translation IC_{50} (μM)	MIC (μM) ATCC 25922
120	a		>100	>100
233	b		>100	>100
121	a		>100	>100
234	b		>100	>100
122	a		>100	>100
235	b		>100	>100
125	a		>100	>100
236	b		>100	>100
124	a		>100	>100
237	b		>100	>100
123	a		>100	>100
238	b		>100	>100

2.2.6 Tier 2 Testing of Selected Gougerotin Analogs

We chose a select group of compounds that show limited activity in the first round of the Tier 1 MIC assay (*E. coli*, *S. aureus*) for testing in the Tier 2 assay. The Tier 1 is designed to identify compounds with antimicrobial activity, while the Tier 2 assay is designed to test the effect of these compounds against strains that have defects in emrE pumps or mexB efflux pumps (strains obtained from Prof. H. Nakaido, UC Berkeley) to detect compounds that may have limited initial activity, but would benefit from redesign. Table 15 shows a representative set of 8 compounds that were tested against three mutant strains. No activity (<7uM) was obtained to justify targeted medicinal chemistry towards efflux pump activity.

Table 15. Biological Activity of Selected Analogs in the Tier 2 Panel.

Compound	D21F2 (μM)	K2M120818 (μM)	Ram 506(μM)
129	>100	50-100	>100
182	>100	50-100	>100
230	12-25	6-12	50-100
218	>100	12-25	>100
230	12-25	6-12	50-100
131	>100	>100	>100
171	25-50	12-25	>100
127	12-25	6-12	50-100

2.2.7 Cytotoxicity Testing

Table 16. Cytotoxicity of Active Analogs

Compound	MTT (μM)
350	>100
351	>100
357f	>100
357h	>100

A selection of compounds, most meeting our requirement of MIC $\leq$ 25uM, was chosen for testing in the MTT assay for cytotoxicity. This testing was done, in part, because early on we had failed to see any difference between eukaryotic and prokaryotic translation inhibition in a representative selection. However, none of these compounds were toxic at concentrations up to 100uM in the cellular assay. The results are shown in

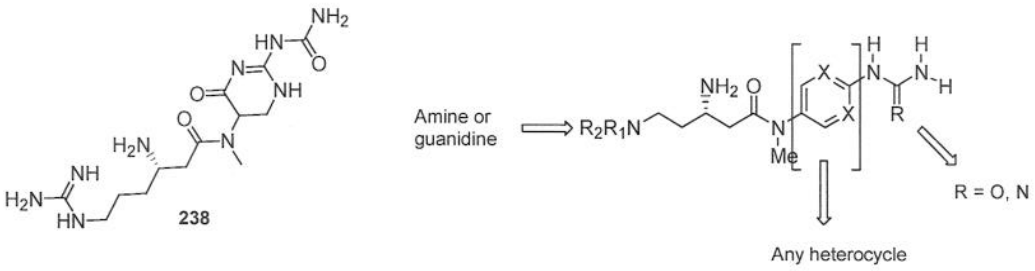
Table 16. Overall, we have not been able to demonstrate sufficient activity to warrant advancing these compounds, as is, into animal models and have discontinued work on this class of analogs.

2.2.8 Sugar-Free Analogs

Using our developed methodology, we prepared a large number of pyranosyl cytosines analogous to the lead structure. All of the compounds prepared were immediately evaluated in both the transcription/translation and antimicrobial assays as described in task 4. The information obtained from these biology experiments was then used to better understand the SAR of the compound series, and facilitates the design of more potent and pharmaceutically acceptable antimicrobial compounds. Among these series is the N-4 aromatic substituted gougerotins. Although, we have prepared and evaluated several hundred analogs and improved the activity several fold over, our best compounds are still approx 10-20 fold less active than what is desired for a successful drug candidate.

However, we did notice at this time that all of our analogs contained the cytidine base and peptidic side-chain separated by a sugar spacer. It was recently reported that analogs of TAN1057 (**238**) had outstanding MIC activity with a broad antibacterial profile.(*25*) Also targeting RNA, it was not difficult to notice that compound **238** (figure 3) was an acyclic pyranosyl cytosine analog. We believe that such acyclic compounds retain the ability to inhibit transcription/translation and have an increased ability to either penetrate the bacterial cell membrane, avoid resistance conferring enzymatic manipulations and/or avoid one or more of the many up regulated small molecule pumps that are characteristic of multi-drug-resistant bacteria. Molecular modeling experiments confirm that analogs such as **238** overlay well with our series of gougerotin analogs, and suggest several intriguing hybrid structures. Therefore, we planned to focus the direction of the gougerotin work on acyclic analogs as indicated in Figure 3, while discontinuing work on the Gougerotin series, per se.

Figure 3. Acyclic Gougerotin Analogs.

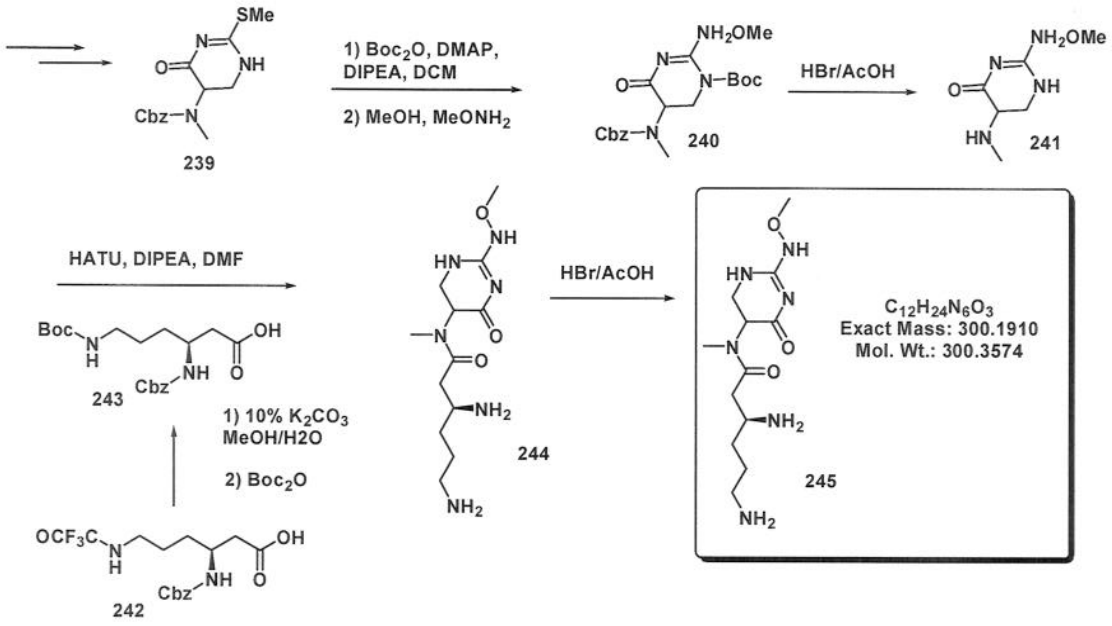


These compounds are interesting from a small molecule – RNA binding perspective. They are low molecular weight (MW = 356) compounds that possess a H bond acceptor – donor motif which is linked by a flexible tether to a distal guanidine. An additional amino group also adorns the flexible linker. By drawing parallels to our 2-aminobenzimidazole series of RNA binders (*vida infra*), the amidinourea probably provides H bond interactions while the positively charged amine and guanidine, provide interactions with the phosphate backbone.

2.2.9 Synthetic Methodology of Acyclic Gougerotin Analogs

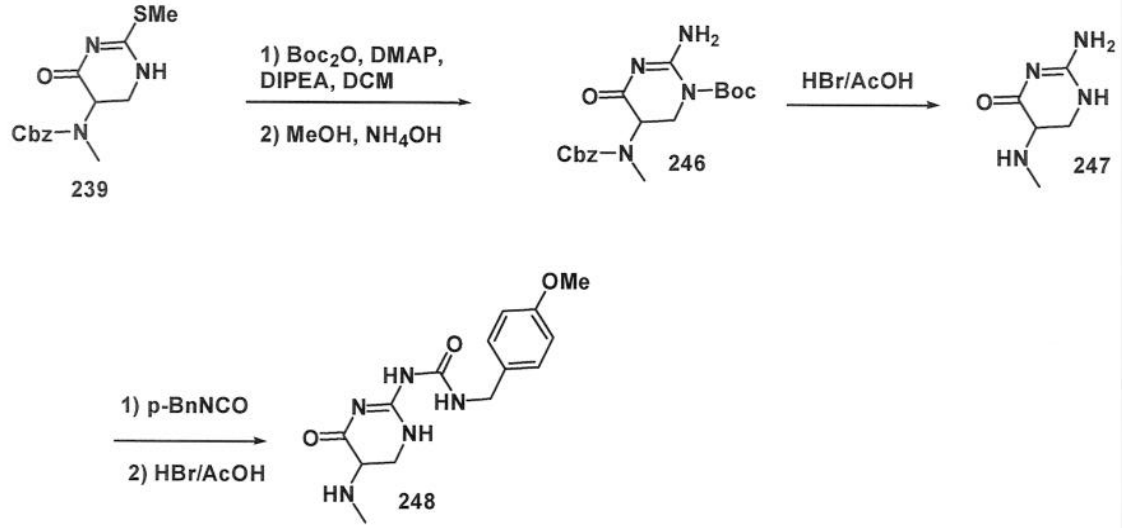
The synthetic methodology we implemented was a modified version of that used by others. Initially we prepared several grams of the methylthio precursor **239**, Scheme 312. This compound could then be converted into its BOC derivative followed by displacement, e.g. methoxyamine as shown in Scheme 12. A key step is the simultaneous deprotection of **240** with HBr/AcOH. This allows us to obtain gram-scale quantities of the coupling precursor **241**, without the need for chromatography. The sidechain, **242**, could be prepared in a multi-gram scale in a one-pot reaction as outlined in the scheme. Coupling was nearly quantitative and deprotection was accomplished in a manner similar to the conversion of **240 → 244**.

Scheme 12. The synthesis of an acyclic gougerotin analogs.



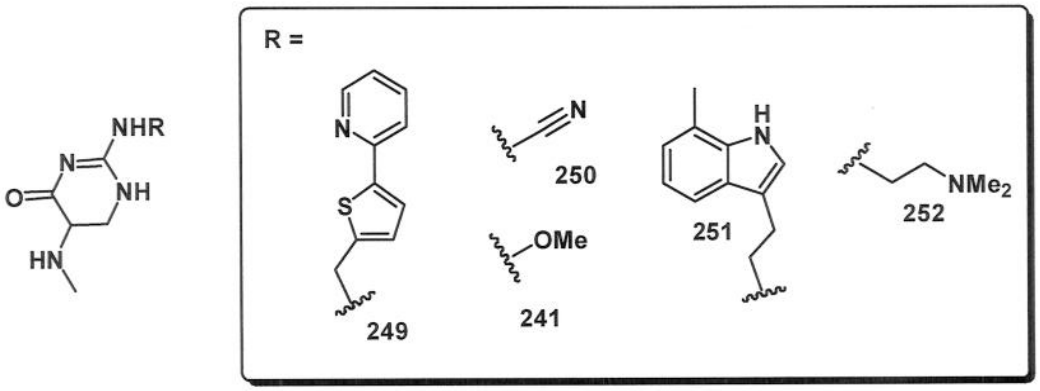
Interestingly, we discovered that we could prepare urea analogs of **241** using our methodology. This is significant in that a urea functionality is present in TAN itself.

Scheme 13. The synthesis of urea acyclic gougerotin precursors.



Several examples of the diverse array of analogs available is shown in Figure 13. We are currently preparing several of these acyclic gougerotin analogs for evaluation.

Figure 4. Examples of analogs that can be prepared.



2.2.10 Biological Evaluation of Acyclic Gougerotin Analogs

Testing for transcription/translation activity, as well as for the minimum inhibitory concentration against a pathogenic strain of *E. Coli* and *S. aureus*, was carried out and, unfortuntely, no significant activity was found for any of the acyclic analogs (Table 17). We have discontinued development of these analogs due to the unanticipated lack of activity. As we stated in our 2003 report, "If a select array of analogs proves to fail the tier I and tier II hurdles as outlined in the original proposal, we will discontinue the development of analogs outlined in Task 1."

While our original work on the pyranosyl cytosines had been submitted for publication to *the Journal of the American Chemical Society,* the reviewers suggested that *Organic Letters* would be a better choice, and this manusctipt is currently under peer review at that journal. A copy is enclosed in the appendix.

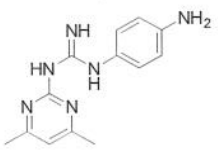
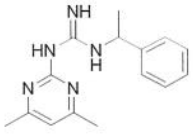
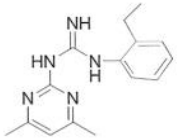
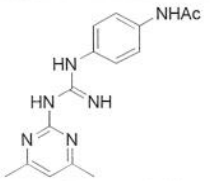
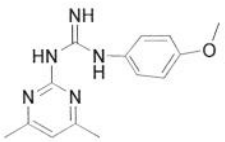
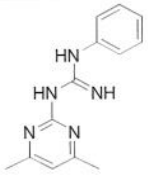
Table 17. Biological Evaluation of Acyclic Gougerotin Analogs

Compound	MIC *E. coli* (μM)	MIC *S. aureus* (μM)
249	>100	>100
250	>100	>100
251	>100	>100
252	>100	>100

Task 2: Lead Optimization of Biaryl Guanidines

As mentioned in our original proposal, the disubstituted guanidine IBIS00372433 (Figure 5) was identified as an inhibitor of cell-free transcription/translation in *E. coli*, as part of a broad based high throughput screening effort of the Ibis library of compounds. Subsequent dose-response experiments indicated this compound was a reasonably potent lead structure, having an IC_{50} of 3.1 μM. Furthermore, in a eukaryotic system, the transcription/translation inhibition the IC_{50} was found to be > 200 μM, indicating a strong selectivity for prokaryotes. The goal of Task 2 is to investigate the SAR of the biaryl guanidine system by systematic modification of the structure. This first stage was designed to provide information regarding the key components required for activity, which is used in turn to guide elaboration into more potent compounds. Further, these structures lacked strong antimicrobial activity despite having good activity in the *in vitro* transcription/translation system, making the achievement of an optimization lead possessing antibacterial activity our second stage goal. In our previous report, we outlined significant progress towards both of these goals, including; the development of a novel benzimidazole core that came out of our guanidine SAR, and the discovery of a candidate that was suitable for initial mouse toxicity testing.

Figure 5. Transcription/translation activity of biaryl guanidines.

			
	IBIS00372433	IBIS00374942	IBIS00375050
T/T IC50 (μM)	3	50-75	75-100
			
	IBIS00374988	IBIS00371656	IBIS00375109
T/T IC50 (μM)	> 100	> 100	> 100

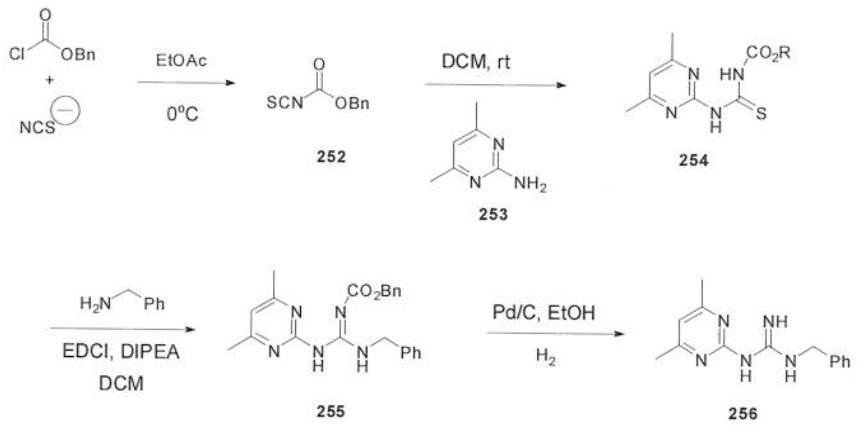
Herein, we outline the sum of progress that we have made in this series.

2.2.11 Synthetic Methods

We have chosen two separate and complementary paths to prepare a large set of guanidines. First, we have developed a highly efficient and parallel solution-phase synthesis which we used to prepare a large number of guanidines. Second, we have implemented a modified solid-phase protocol for the preparation of disubstituted guanidines. The route that was employed is an improvement of a published synthesis of disubstituted guanidines.

The first of these procedures is demonstrated in Scheme 14. Treatment of benzyl chloroformate with powdered potassium isothiocyanate in ethyl acetate at 0C for 12 hours gives a mixture of desired product **252** and benzylthiocyanate (not shown).(*28*) This reagent, as a crude mixture, is then treated with an appropriate amine, such as aminodimethylpyrimidine **253** in dichloromethane at room temperature for 24 hours. The resultant thiourea **254** can be purified by column chromatography and split into several portions for parallel synthesis.

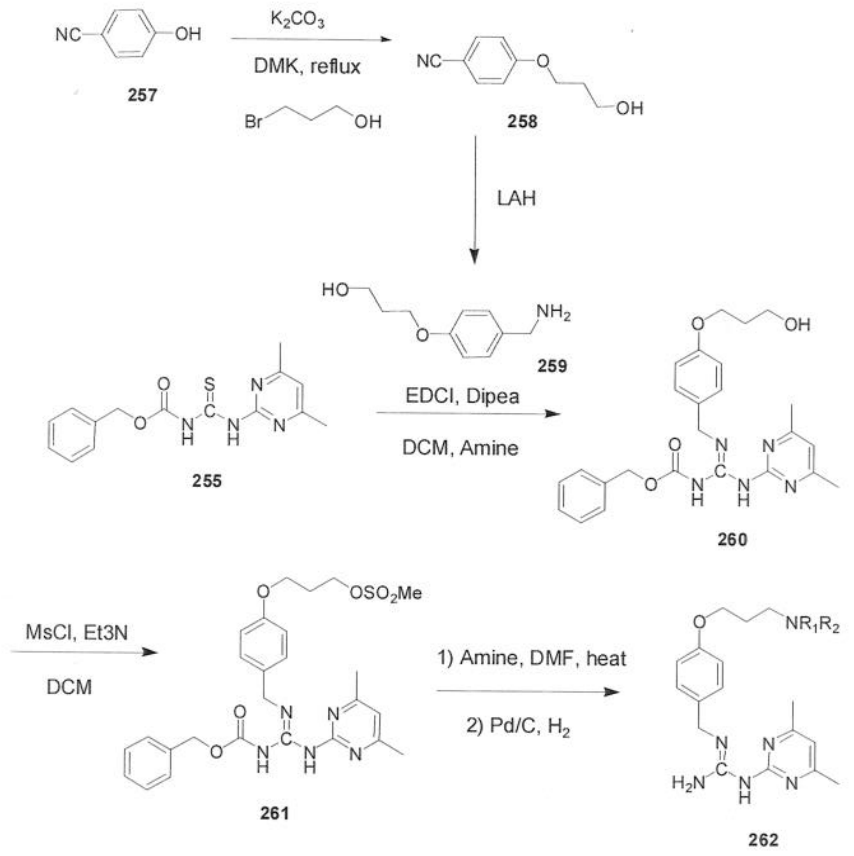
Scheme 14. Solution-Phase Synthesis of Guanidines.



Treatment of the thiourea with an amine, in this case benzylamine, in the presence of standard coupling conditions gives a high yield of the substituted guanidine **255** which is obtained as a pure compound after parallel flash chromatography using a Jones Flashmaster II. The final compound can then be deprotected under catalytic

hydrogenation to give pure **256** without the need for further purification. Over one hundred compounds were prepared using this method giving 10-30 mg of each compound with a purity of >90% (as determined by HPLC, using ELSD detection).

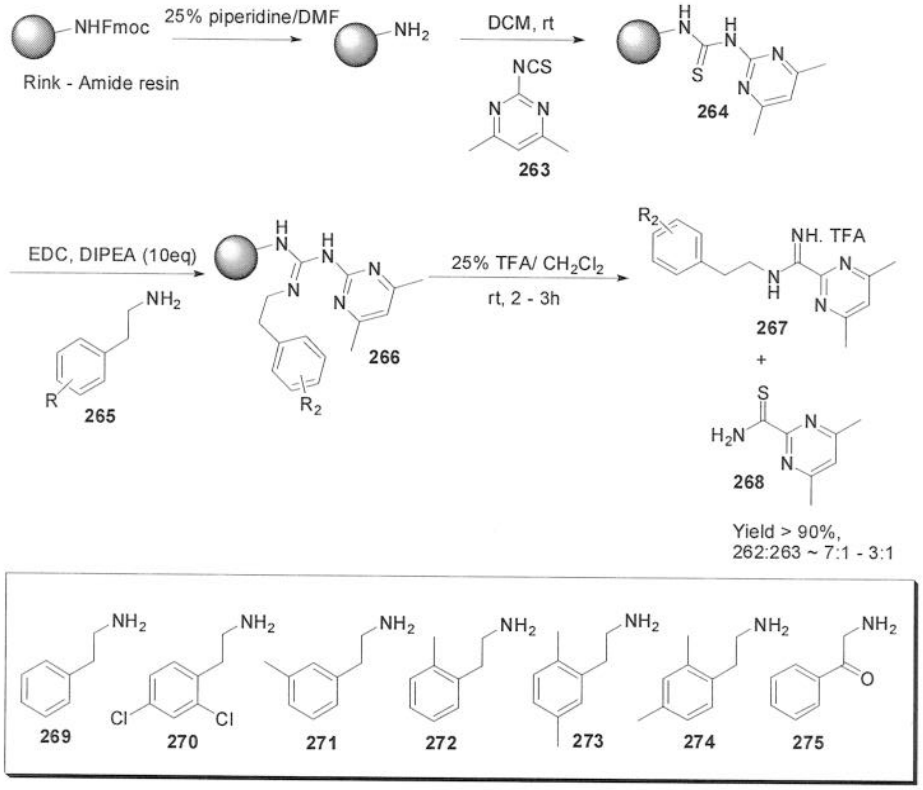
Scheme 15. Synthesis of Guanidines with Amino Head Groups.



To obtain guanidines tethered with side chains at the terminal hydroxy group, we first began by preparing the amine **259** using an alkylation, reduction strategy. This amine could then be coupled to thiourea **255** as previously described to give the protected guanidine **259**. Mesylation of the free hydroxy group, followed by displacement with an amine and deprotection gave the final bi–substituted guanidine **262**.

Next our attention turned to the employment of an improvement of a published(*29-35*) solid-phase syntheses of disubstituted guanidines and is outlined in Scheme 16. The original procedure used PS-Rink amide resin and utilized DIC as the activating agent for coupling the aliphatic amine with the resin bound thiourea. The authors found that this procedure worked best when the aromatic ring was substituted with electron withdrawing groups and when the coupling was carried out at 50 °C in $CHCl_3$.

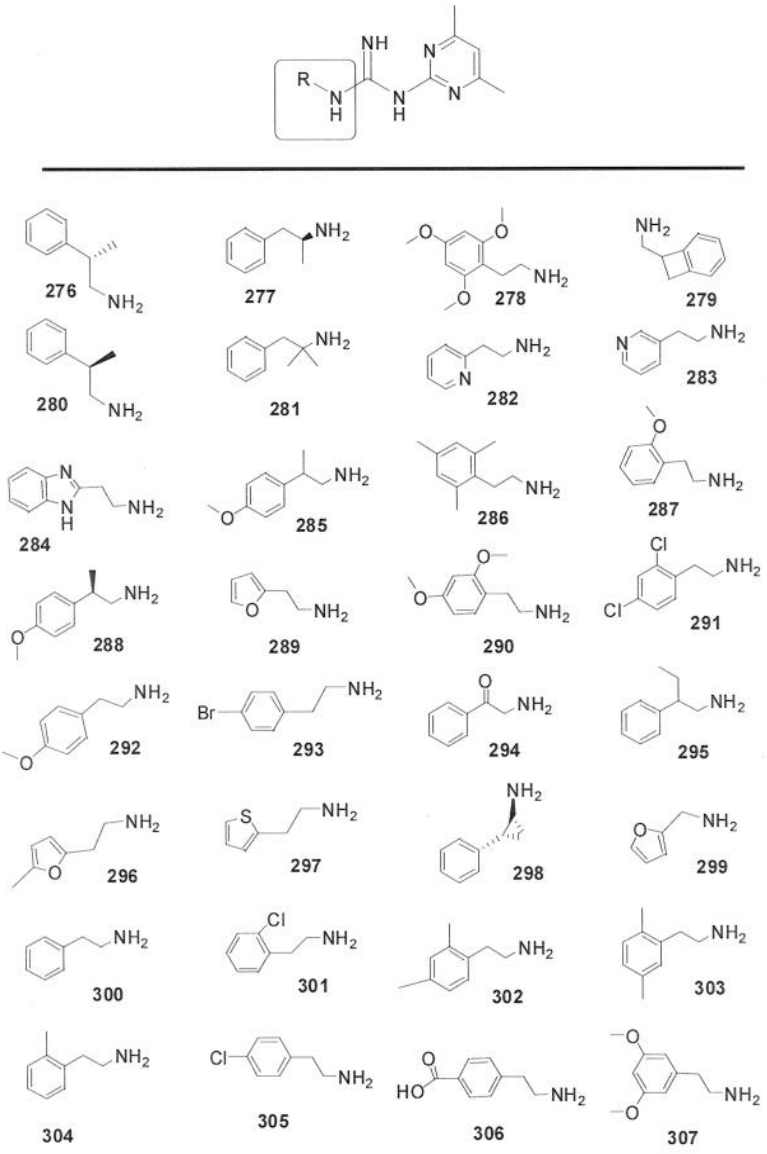
Scheme 16. Solid-Phase Synthesis of Guanidines.



We subsequently modified the procedure by replacing PS-Rink with ArgoGel-Rink and DIC with EDC in CH_2Cl_2 at 40°C. In all cases the products **269-275** were obtained in excellent yield (typically >90%) but they were contaminated with differing amounts of the unreacted thiourea **268**. We attempted to improve the coupling efficiency by washing the resin **266** and repeating the coupling procedure a second time but were unable to push the reaction to completion. However, the cleavage of guanidines from the resin was quite

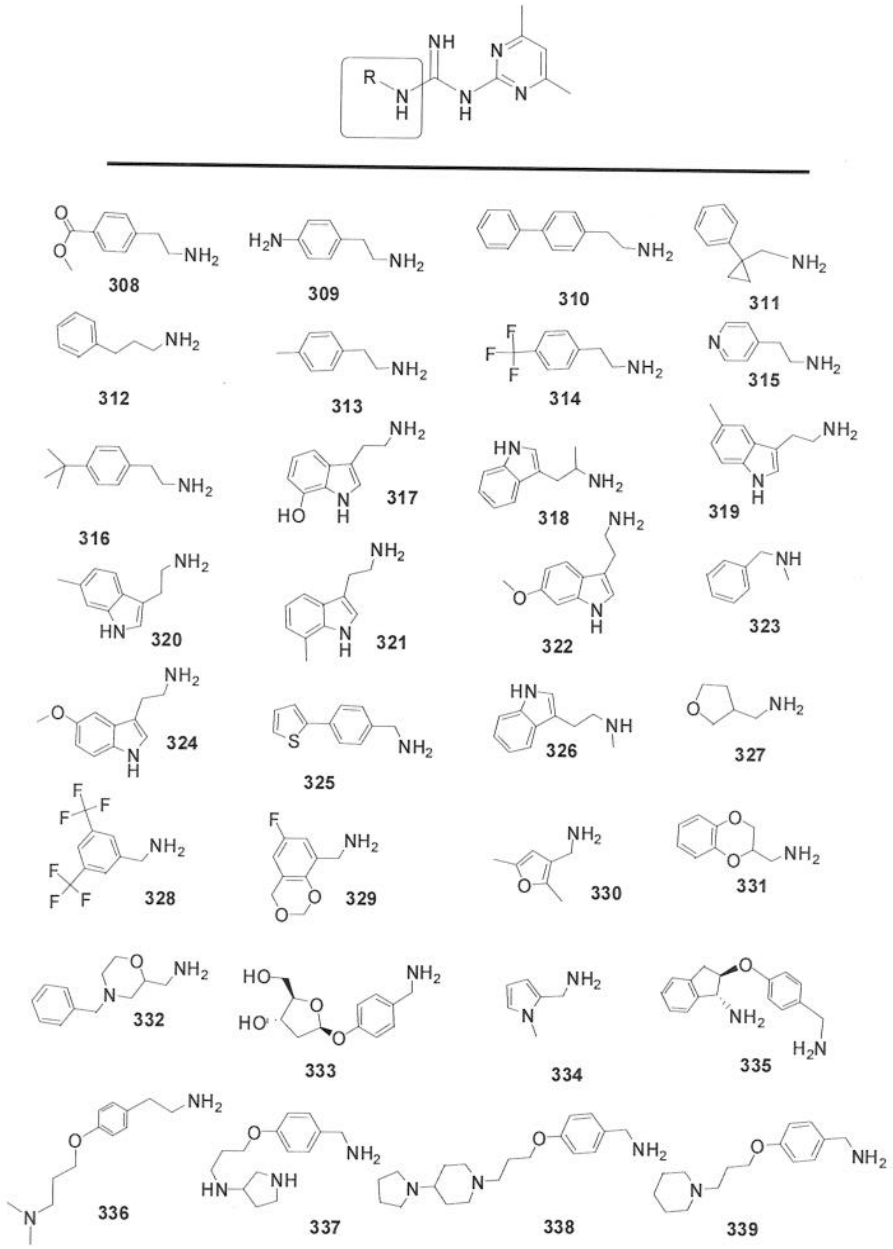
efficient using 25% TFA/ CH_2Cl_2, with almost 90% of product **267** being cleaved after the first TFA treatment.

Figure 6. Aromatic Group Modified Compounds Prepared.



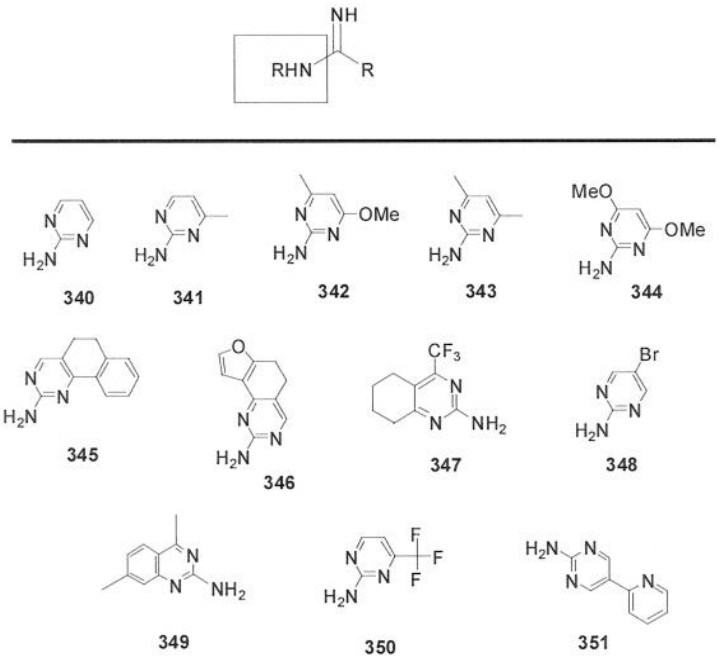
To ensure complete conversion, we cleaved the resins a second time with 100% TFA. Typically, the second cleavage provided about 8 mg of material as compared to 80 mg obtained from the first cleavage (starting with 500 mg of resin, loading 0.35 mmol/gm). The crude guanidine TFA salts were then purified by preparative HPLC to give pure products for testing in T/T and MIC. The analogs made according to one or more of these methods is shown in Figures 6-8.

Figure 7. Aromatic Group Modified Compounds Prepared (Continued).



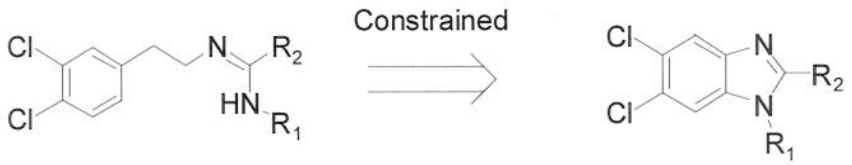
Specifically, variations were made in a combinatorial parallel fashion. Figures 6 and 7 illustrate the modifications used on the aromatic region, while Figure 8 illustrates modifications made to the heterocyclic portion. In total over 500 compounds were made and tested in the assays as described in Task 4.

Figure 8. Heterocyclic Modified Compounds Prepared.



Finally, a series of benzimidazoles, which can be viewed as constrained aryl guanidines were prepared (Figure 9). The details are provided in the unpublished manuscript titled, "2-Piperidin-4-yl-benzimidazoles with Broad Spectrum Antibacterial Activities" in addition to later in this report.

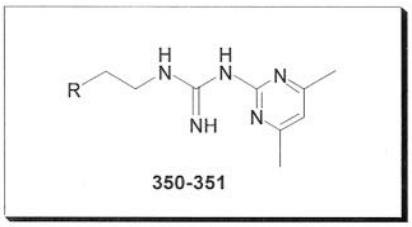
Figure 9. Constrained Guanidine Analogs.



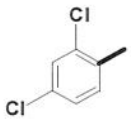
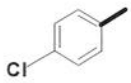
2.2.12 Biological Evaluation of the Biaryl guanidines and Related Analogs

The biological evaluation of over 400 guanidine analogs is completed. Testing for transcription/translation activity, as well as for the minimum inhibitory concentration against a pathogenic strain of *E. Coli*, was carried out. Among all of these analogs, only a very small number showed any significant transcription/translation activity and an even smaller subset showed any MIC activity. One noticeable exception was the dichloroguanidines. As shown in figure 10, compounds **355** and **356** have modest antibacterial activity. This prompted us to explore the synthesis and evaluation of a series of dichlorobenzimidazoles, which can be thought of as constrained aryl guanidines (Figure 10.)

Figure 10. Biological Evaluation of the Dichloroguanidines.



350-351

Compound	R	MIC *E. Coli. (uM)* ATCC 25922	MIC *S. Aureus* (μM) ATCC 13709
355		50-100	25-50
356		50-100	>100

This work is described in the manuscript attached as an appendix, and the detailed synthesis and biological evaluation is reported therein. The biological data is also included in Tables 18a-21. Just to mention the highlights; a series of compounds were discovered which showed activity against representative gram positive and negative organisms. From this series, a subset of compounds were evaluated for activity against a range of bacteria, and ultimately compounds **357f** and **35fh** were selected for further study, including preliminary animal toxicity studies as outlined in Task 4.

Table 18a. Inhibitory Effects of Benzimidazoles on S. aureus and *E. coli* Growth and Bacterial Transcription/Translation.

Compound	ATCC 13709 S.aureus MIC (μM)	ATCC 25922 E.coli MIC(μM)	T/T IC_{50} MIC (μM)
354a	>100	>100	>100
354b	>100	>100	>100
354c	50-100	50-100	>100
354d	50-100	50-100	>100
354e	>100	>100	>100
354f	50-100	>100	>100
354g	>100	>100	>100
357a	6-12	12-25	>100
357b	3-6	6-12	>100
357c	6-12	12-25	12
357d	12-25	50-100	20
357e	6-12	25-50	50
357f	6-12	12-25	>100
357g	6-12	6-12	>100
357h	3-6	12-25	10
357i	6-12	12-25	>100
357j	12-25	12-25	>100
357k	6-12	12-25	25
357l	12-25	12-25	>100
357m	12-25	12-25	>100
357n	6-12	12-25	>100
357o	6-12	12-25	>100
357p	6-12	50-100	60
357q	12-25	12-25	>100
357r	6-12	6-12	>100

Figure 11. Biological Evaluation of Dichlorobenzimidazoles

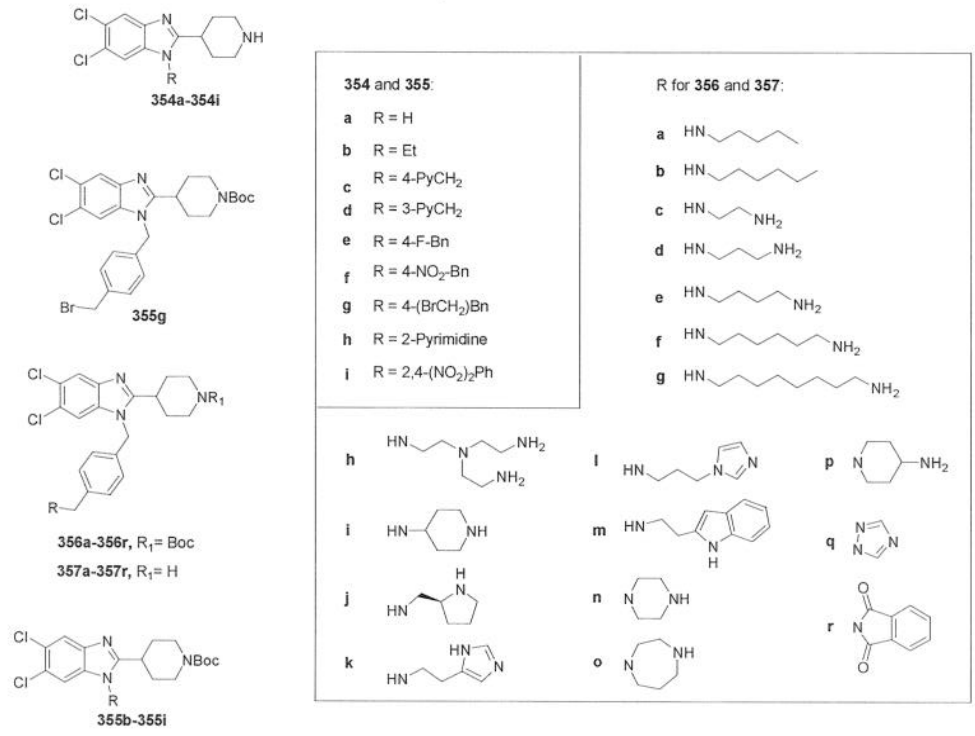


Table 18b. Minimal Inhibitory Concentrations (MIC, μM) of Benzimidazoles against Bacteria.

Compound	SA1	EH2	SP4	SP6	EC2	VP8	KP1	PA2
357a	6-12	1-3	3-6	12-25	12-26	25-50	6-12	25-50
357b	3-6	1-3	3-6	6-12	6-12	12-25	6-12	25-50
357c	6-12	3-6	6-12	12-25	12-25	25-50	12-25	12-25
357d	12-25	6-12	6-12	25-50	50-100	NT	25-50	25-50
357e	6-12	3-6	6-12	25-50	25-50	NT	25-50	25-50
357f	6-12	1-3	3-6	12-25	12-25	25-50	12-25	25-50
357g	6-12	1-3	3-6	6-12	6-12	12-25	6-12	25-50
357h	3-6	3-6	3-6	6-12	12-25	25-50	12-25	12-25
357i	6-12	3-6	6-12	12-25	12-25	25-50	12-25	12-25
357k	6-12	3-6	6-12	12-25	12-25	25-50	12-25	25-50
357n	6-12	3-6	6-12	12-25	12-25	12-25	6-12	12-25
357o	6-12	3-6	6-12	12-25	12-25	25-50	12-25	25-50
357p	6-12	3-6	6-12	25-50	50-100	25-50	25-50	12-25
357r	6-12	3-6	12-25	6-12	6-12	25-50	25-50	12-25

[a] SA1: *S. aureus* 13709; EH2: *E. hirae* 29212; SP4: *S. pyogenes* 49399; SP6: *S. pneumoniae* 6303; EC2: *E. coli* 25922; PV8: *P. vulgaris* 8427; KP1:
K. pneumoniae 13383; PA2: *P. aeruginosa* 25416; CA1: *C. albicans* 10231; NT: Not tested.

Table 19. Minimal Inhibitory Concentrations (MIC, μM) of Benzimidazoles against *Enterococcus*.

Compound	*E. faecalis* ATCC11823	*E. faecalis* ATCC 23241	*E. faecalis* ATCC 4200	*E. faecalis* ATCC 49757
357a	3-6	6-12	6-12	6-12
357b	3-6	3-6	3-6	3-6
357c	6-12	12-25	12-25	12-25
357d	12-25	25-50	25-50	12-25
357e	12-25	12-25	12-25	12-25
357g	6-12	NT	25-50	50-100
357I	12-25	12-25	12-25	12-25
357l	6-12	12-25	12-25	12-25
357n	12-25	6-12	12-25	12-25
357o	12-25	12-25	12-25	6-12
357p	12-25	12-25	12-25	12-25
357r	3-6	6-12	6-12	6-12

Table 20. Minimal Inhibitory Concentrations (MIC, μM) of Benzimidazoles against *Enterococcus*.

Compound	*E. faecalis* ATCC 828	*E. faecium* ATCC 6569	*E. faecium* ATCC 882	*E. hirae* ATCC 29212
357a	6-12	3-6	6-12	1-3
357b	3-6	3-6	3-6	1-3
357c	12-25	6-12	12-25	3-6
357d	12-25	12-25	25-50	6-12
357e	12-25	12-25	12-25	3-6
357g	50-100	12-25	NT	1-3
357I	12-25	12-25	12-25	3-6
357l	6-12	6-12	12-25	3-6
357n	6-12	6-12	6-12	3-6
357o	12-25	6-12	12-25	3-6
357p	12-25	6-12	12-25	3-6
357r	6-12	3-6	6-12	3-6

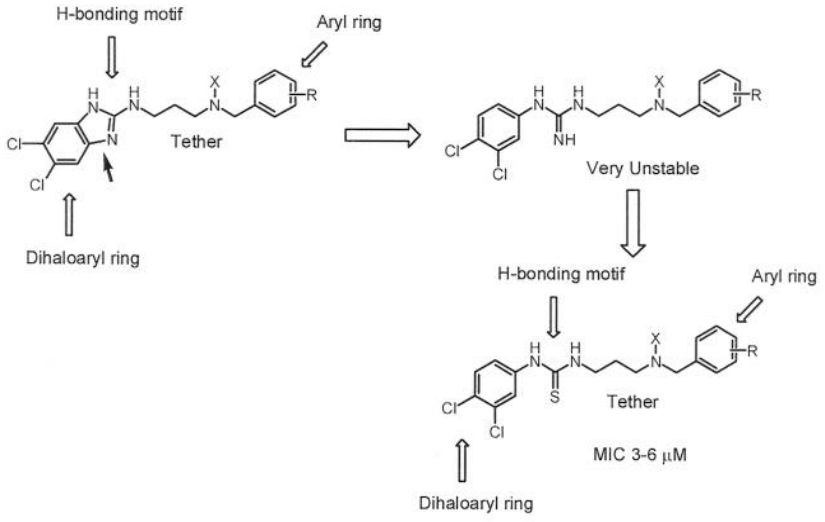
Table 21. Cytotoxicity of Active Analogs

Compound	MTT (μM)
350	>100
351	>100
357f	>100
357h	>100

A selection of compounds, meeting our requirement of MIC $\leq$ 25 μM, was chosen for testing in the MTT assay for cytotoxicity. None of these compounds were toxic at concentrations up to 100 μM. The results are shown in Table 21. These compounds represent selectivites in excess of 10 fold and were candidates to take on into animal toxicity studies (*vida infra*).

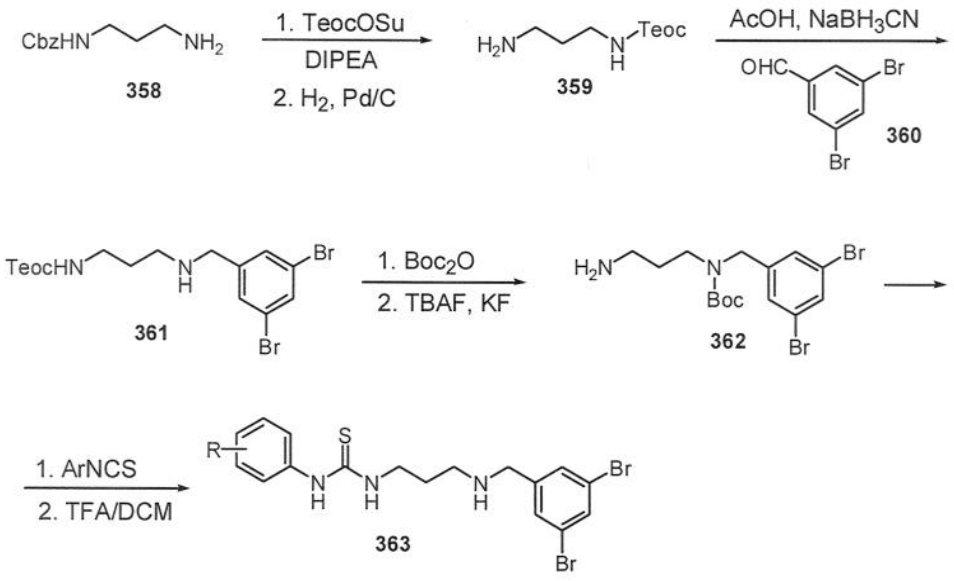
A further expansion of the biaryl guanidine SAR plan is illustrated in Figure 12. Synthetically, we required access into tethered heteroaromatic areas while maintaining the hydrogen bonding motif in both the areas and benzimidazole analogs. We have previously reported the synthesis of several benzimidazoles in addition to several ureas in our 2003 annual report, and herein we report the progress made in our analog design and synthesis.

Figure 12. General SAR Plan.

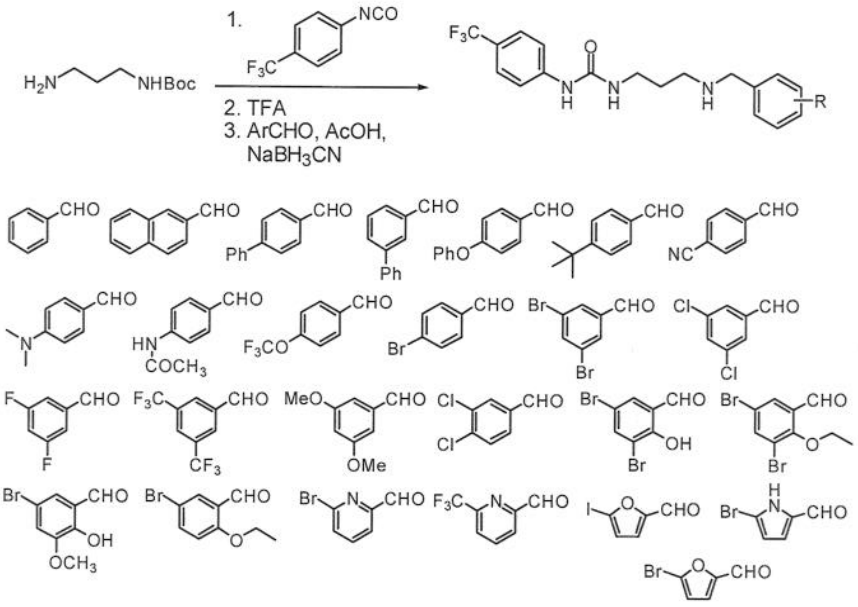


As illustrated in Scheme 17, the protecting group of a commerically available Cbz-protected diamine was swapped with a Teoc. A reductive amination of the new diamine gave compound **360**. Subsequent protection, treatment with a isothiocyanate and final deprotection gave compound **363**. This methodology was found to be of general synthetic utility, and was used to prepare the thiourea analogs. As shown in Scheme 18, a wide variety of ureas were prepared using similar methodology.

Scheme 17. Example of analog.



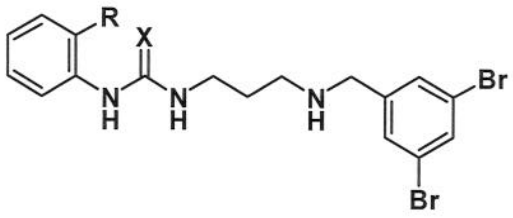
Scheme 18. Several aldehydes used to accomplish the reductive aminations.



2.2.13 Biological Evaluation of the Biaryl guanidines and Related Analogs

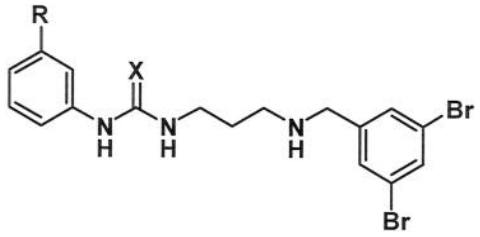
The biological evaluation of several urea, thiourea and benzimidazole analogs have been completed. Testing for transcription/translation activity, as well as for the minimum inhibitory concentration against a pathogenic strain of *E. Coli* was carried out.. As indicated in our previous report, the di-halogenated tethered analogs were among the most active. We chose to initially focus on the dibromo analogs to explore the SAR. The first set of analogs contains an orto-substituent at the aniline portion. Both area and thiourea analogs were prepared. As can be seen in Figure 13, none of these were very active and did not meet our workflow criteria for advancement.

Figure 13. Ortho-substituted analogs.



R	X	*S. aureus*	*E. coli*
Cl	S	12-25	25-50
F	S	25-50	50-100
OMe	S	>100	>100
SMe	S	50-100	50-100
SMe	O	50-100	50-100
OMe	O	25-50	50-100

Figure 14. Meta-substituted analogs.

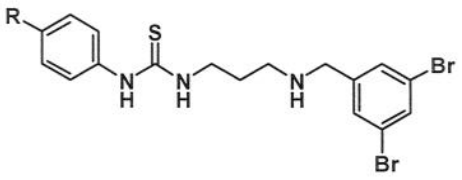


R	X	*S. aureus*	*E.coli*
Cl	S	6-12	12-25
F	S	12-25	25-50
OMe	S	25-50	50-100
Br	S	6-12	>100
CF3	S	6-12	50-100
CF3	O	6-12	25-50
Zyvox	-	3-6	>100

On the other hand, the meta-substituted analogs in both the urea and thiourea series showed significant activity. In fact, we were approaching the levels of the highly-successful, commercial drug Zyvox, and were beginning to even show gram negative activity.

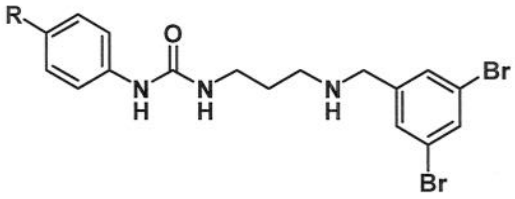
Next we began to look at the para-substituted analogs (Figure 15) and saw similar results. However, the para-substituted urea analogs looked even more promising. As outlined in Figure 16, the CF3-substituted analog showed promise as it was identical to Zyvox against S. aureus and had a hint of activity against pathogenic E. coli. This was indeed promising and prompted us to further explore substitutions of the benzylic portion by holding the para-CF3 substituted portion constant.

Figure 15. Para-substituted thiourea analogs.



R	*S. aureus*	*E. coli*
OMe	25-50	50-100
Cl	6-12	25-50
F	12-25	25-50
CH3	12-25	>100
Br	12-25	>100
NO2	12-25	>100
CN	25-50	>100
I	12-25	>100
CF3	6-12	50-100
Zyvox	3-6	>100

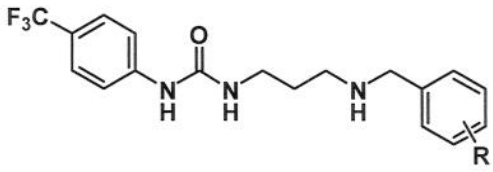
Figure 16. Para-substituted urea analogs.



R	*S. aureus*	*E. coli*
SMe	6-12	12-25
OCF3	6-12	25-50
Cl	6-12	12-25
CF3	3-6	12-25
Zyvox	3-6	>100

As outlined in Figure 17 both the dichloro and dibromo-derivatives were the most active. The other halogens or the dimethoxy failed to demonstrate any significant activity. This further prompted us to prepare a series of mono-substituted analogs.

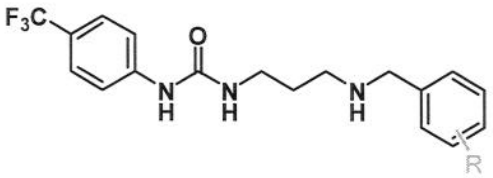
Figure 17 Para-trifluoromethyl-substituted urea analogs.



R	*S. aureus*	*E. coli*
3,5-dibromo	3-6	12-25
3,5-difluoro	50-100	25-50
3,5-dichloro	6-12	12-25
3,5-di CF3	50-100	50-100
3,5- dimethoxy	25-50	50-100
3,4-dichloro	3-6	12-25
Zyvox	3-6	>100

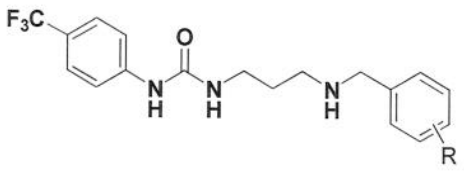
The results are shown in Figure 18 As ascertained before, the para-substituted compounds were significantly more active than the ortho or meta-substituted compounds. In particular, the 4-phenyl, 4-phenoxy, 4-triflouromethyl and 4-butyl were the most active, showing both good gram positive and the best gram negative seen at date. Furthermore, the active compounds were carried on to a larger panel of organisms as outlined in our revised workflow scenario. Several compounds demonstrated good MIC against different strains of gram positive and gram negative organisms, and two compounds (i.e. the phenyl, phenoxy and dibromo) were chosen to take on to the cell-based in vitro assay as outlined in the original and revised workflows. Only the phenyl and dibromo qualified to pass on to the next stage, animal toxicity testing, as the phenoxy was too toxic and had too low a therapeutic index as determined by the criteria set forth in our workflow (Figure 19.

Figure 18 Para-trifluoromethyl-substituted urea analogs.



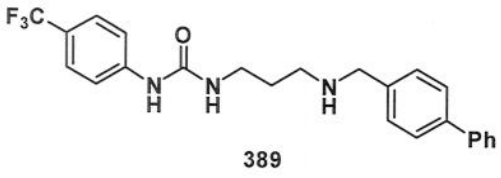
R	*S. aureus*	*E. coli*
H	50-100	50-100
naphthyl	12-25	12-25
2-OCF3	12-25	12-25
3-phenyl	6-12	6-12
4-phenyl	3-6	6-12
4-phenoxy	3-6	6-12
4-OCF3	3-6	6-12
4-*t*-butyl	3-6	6-12
4-bromo	12-25	12-25
4-dimethylamino	12-25	25-50
4-NHCOCH3	>100	>100
Zyvox	3-6	>100

Figure 19 Selected analogs tested against a broad panel.



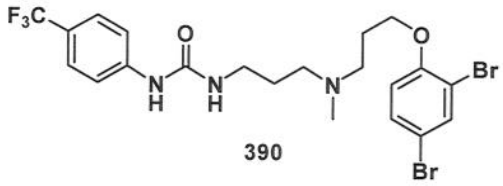
	3,5-diBr	2-OEt-3,5-diBr	4-Ph	4-OPh	4-tButyl	Zyvox
S.aureus	3-6	3-6	3-6	3-6	3-6	3-6
S.pyogenes	6-12	3-6	3-6	3-6	3-6	1.5-3
E.faecalis	6-12	6-12	>100	3-6	6-12	3-6
E.faecium	>100	6-12	>100	>100	3-6	>100
E.coli	12-25	12-25	6-12	6-12	6-12	>100
K.pneumoniae	6-12	3-6	6-12	6-12	3-6	25-50
P.vulgaris	12-25	12-25	12-25	12-25	12-25	12-25
P.aeruginosa	>100	>100	>100	>100	NT	>100

Figure 20 Cell-Based MTT toxicity results.



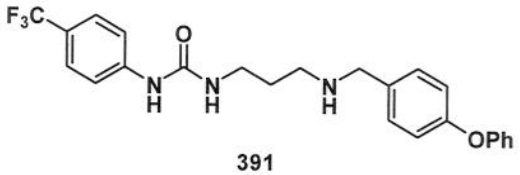
389

MTT TC_{50} = 50-100 mM (24 h), 50mM (48 h)



390

MTT TC_{50} = (24 h, 48 h) = 50-100 mM



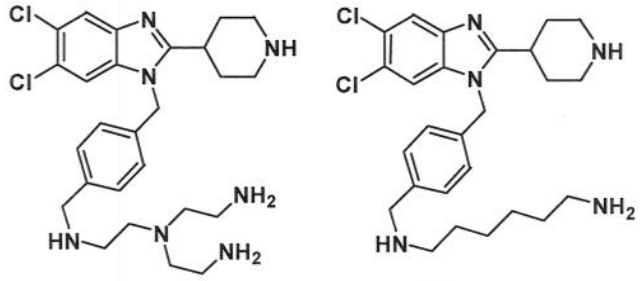
391

MTT TC_{50} = = 25-50 mM (24 h), 13-25 (48 h)
Failed Workflow

The two compounds, **389 a**nd **390**, met our requirement of MIC ≤ 25 μM and MTT selectivity >10 fold, and were chosen for testing in animal models of toxicity and protection. These experiments are outlined in Task 4.

Additionally, the benzimidazoles **392** and **393 (Figure 21)**, were tested in animal models of toxicity and protection and are outlined in Task 4. Finally, we report the publication of "Synthesis and biological evaluations of novel benzimidazoles as potential antibacterial agents." *Bioorganic & Medicinal Chemistry Letters* **2004,** 14, (5), 1217-1220, and "2-Piperidin-4-yl-benzimidazoles with broad spectrum antibacterial activities." *Bioorganic & Medicinal Chemistry Letters* **2003,** 13, (19), 3253-3256 which are included as appendix 3 and 4.

Figure 21. Benzimidazoles advanced to Task 4.



2.3 Task 3: Lead Optimization of Biaryl Amidrazones

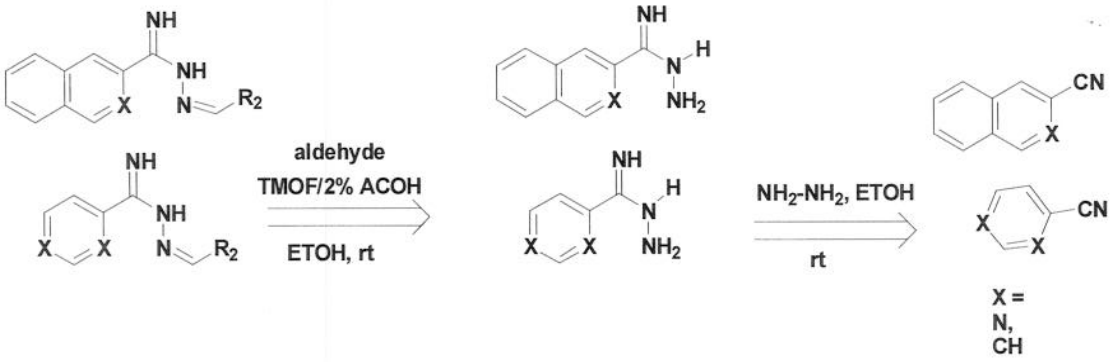
A bis-aryl substituted amidrazone IBIS00370479 was identified as an inhibitor of cell-free transcription/translation in *E. coli*, as part of a high throughput screening effort of the Ibis library of compounds. Subsequent dose-response experiments indicated this compound was a very potent lead structure, having an IC_{50} of 0.4 μM. While this was the only amidrazone in the screening set, some preliminary SAR was evident, as several related acyl-hydrazones also showed activity. It is also noteworthy that there are scattered literature reports of certain bis-aryl substituted amidrazones having antibacterial,(*2, 36*) antimycobacterial,(*37-39*) and antifungal(*40*) activities. Despite this interesting activity, the compounds have been relatively poorly investigated, and only a few analogs have been prepared. This suggests that an extensive lead optimization effort may well produce an extremely broad-spectrum antimicrobial development candidate.

In the last report, we had indicated that work in this area was to begin shortly. Herein we are pleased to report the preparation and biological evaluation of some 40 analogs.

2.3.1 Synthesis of Biaryl Amidrazones

The synthesis of the Amidrazones follows the general scheme outlined in scheme 19. Typically, an aromatic nitrile is treated with hydrazine in ethanol to give the caroboxamidrazone. After condensation with an aldehyde, the corresponding final product is isolated as a solid suitable for biological testing.

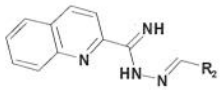
Scheme 19. General Synthesis of Amidrazones.

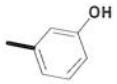
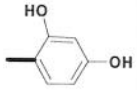
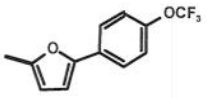
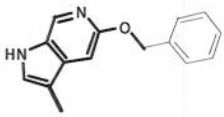
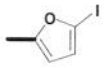
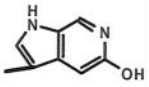


2.3.2 Biological Evaluation of Biarylamidrazones

The biological evaluation of several amidrazone analogs have been completed. Testing for transcription/translation activity, as well as for the minimum inhibitory concentration against a pathogenic strain of *E. Coli* was carried out. Unfortunately, among the most active was the lead compound identified in our original proposal.

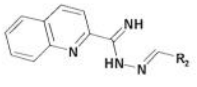
Figure 22. Quinoline Substituted Amidrazones.



R	Trans/ Trans IC50 (uM)	MIC (uM) E. Coli	S. Aureus
	>50	>100	>100
	>50	>100	>100
	>50	>100	>100
	>50	>100	>100
	18	>100	>100
	>50	>100	>100
	>50	>100	>100

Likewise, no compounds that passed Tier 1 MIC screening were discovered and therefore, we did not advance any amidrazones into animal studies. The data and compounds tested can be found in **Figures 23-26**.

Figure 23. Quinoline Substituted Amidrazones.



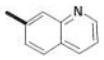
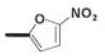
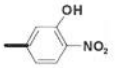
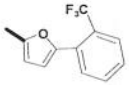
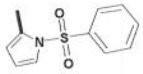
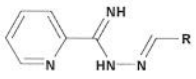
R	Trans/ Trans IC50 (uM)	MIC (uM) E. Coli	S. Aureus
	>50	>100	>100
	>50	>100	>100
	40	>100	>100
	45	>100	>100
	>50	>100	>100
	45	>100	>100
	>50	>100	>100

Figure 24. Pyridine Substituted Amidrazones.



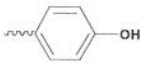
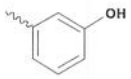
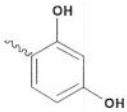
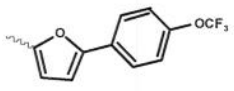
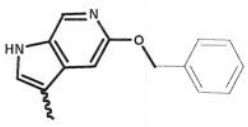
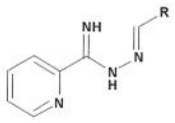
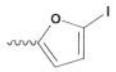
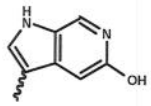
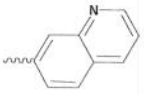
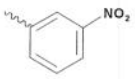
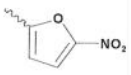
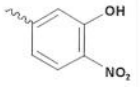
R	Trans/ Trans IC50 (uM)	MIC (uM) E. Coli	S. Aureus
	5.5	>100	>100
	0.5	>100	>100
	19	>100	>100
	>50	>100	>100
	18	>100	>100

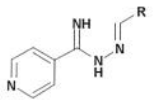
Figure 25. Pyridine Substituted Amidrazones.

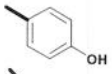
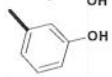
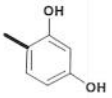
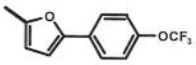
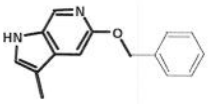
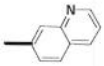
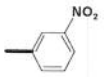
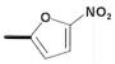
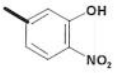


R	Trans/ Trans IC50 (uM)	MIC (uM) E. Coli	S. Aureus
	>50	>100	>100
	>50	>100	>100
	>50	>100	>100
	>50	>100	>100
	>50	>100	>100
	20	>100	>100

Since we have not been able to successfully discover an amidrazone with both transcription/translation inhibitory activity and significant antibacterial activity, we will conclude Task 3 at this stage.

Figure 26. *p*-Pyridine Substituted Amidrazones.



R	Trans/ Trans	MIC (uM)	
	IC50 (uM)	E. Coli	S. Aureus
	>50	>100	>100
	>50	>100	>100
	>50	>100	>100
	>50	>100	>100
	>50	>100	>100
	25	>100	>100
	>50	>100	>100
	Not Avail	>100	>100
	25	>100	>100
	40	>100	>100

2.4 Task 4: In vitro and In vivo Screening of Active Compounds

2.4.1 Inhibition of Prokaryotic and Eukaryotic Translation

The results of this assay can be found in the tables throughout this annual report. This assay was run at Isis Pharmaceuticals in the following manner: The DNA template, pBest Luc ™ (Promega), is a plasmid containing a reporter gene for firefly luciferase fused to a strong *tac* promoter and ribosome binding site. Messenger RNA from 1 μg pBestLuc will be transcribed and translated in E. coli S30 bacterial extract (purchased from Prof. W. Hatfield, UCI) in the presence or absence of test compound. Compounds are tested in a black 96 well microtiter plate with an assay volume of 35 μL. Each test well contains: 5 μL test compound, 13 μL S30 premix (Promega), 4 μL 10x complete amino acid mix (1 mM each), 5 μL *E. coli* S30 extract and 8 μL of 0.125 μg/μL pBest Luc™. The transcription/translation reaction is incubated for 35 minutes at 37°C followed by detection of functional luciferase with the addition of 30 μL LucLite™ (Packard). Light output is quantitated on a Packard TopCount for 30 sec. Selected compounds with an $IC_{50} \leq 5$ μM will be tested in a eukaryotic translation assay based on rabbit reticulocyte lysate (Promega) and a luciferase mRNA with an EMCV promoter.

2.4.2 Measurement of Antimicrobial Activity.

The results of this assay can be found in the tables throughout this annual report. This assay was run at Isis Pharmaceuticals in the following manner: All compounds were tested in bacterial growth-inhibition assays as they are identified in the transcription/translation assay. We currently perform growth-inhibition assays on a panel of up to 12 clinically relevant bacteria in the Ibis BSL-2 facility. Select compounds that show activity in the Tier 1 MIC assay against two strains (*E. coli, S. aureus*) will be subsequently tested in the entire Tier 1 assay. Selected examples with limited activity will be tested in the Tier 2 panel. The Tier 1 is designed to identify compounds with antimicrobial activity against clinically relevant strains of bacteria, the Tier 2 assay is designed to test the effect of these compounds against strains that have defects in emrE pumps or mexB efflux pumps (strains obtained from Prof. H. Nakaido, UC Berkeley) to detect compounds that may have limited initial activity, but would benefit from redesign.

Figure 27. Tier 1 and Tier 2 Panels.

Tier 1		Tier 2	
Organism	ATCC Number	Organism	ATCC Number
*S. aureus***	13709	D21F2 LPS Mutant	nr**
E. hirae	29212		
S. pyogenes	49399	K2M120818 Pump Mutant	nr**
S. pneumoniae	6303		
*E. coli****	25922	Ram 506 Pump Mutant	nr**
P. vulgaris	8427		
K. pneumoniae	13383		
P. aeruginosa	25416		
E. facalis	11823*		
E. faecium	6569		
C. albicans	10231		

* alternatively; ATCC 23241, 4200, 49757, 828
**not registered. see. Journal of Bacteriology, June 200, p.3142-3150 (and references cited therein)
***Tier 1 initial screening organisms

MIC testing was carried out nearly identical to the "Method of Dilution Antimicrobial Susceptibility Tests for Aerobic Bacteria" (as per NCCLS Document M7-A6, Volume 23, No. 2 January 2003).

Specifically, the MIC assays are carried out in 150 μL volume in duplicate in 96-well clear flat-bottom plates. Bacteria from a frozen stock in broth containing 15% glycerol is inoculated into appropriate media (see list below) and grown 6 hours at 37°C. An aliquot of this bacterial suspension (10 uL) is then inoculated into 10 mL media and grown 20-24 hours at 37°C. Bacteria from this overnight culture is added to a solution of

test compound in 1% DMSO in water. Final bacterial inoculum is approximately 10^3 CFU/well. The percentage growth of the bacteria in test wells relative to that observed for a control well containing no compound is determined by measuring absorbance at 595 nm (A_{595}) after 20-24 hours at **37°C** (this constitutes a deviation from NCCLS protocol incubation, where they recommend` 35°C). The MIC is determined as a range of concentration where complete inhibition of growth is observed at the higher concentration and bacterial cells are viable at the lower concentration.

Table 22. Strains used in Tier II Screening.

Bacterial Strain	ATCC	Media
S. aureus	13709	Mueller Hinton Broth (unsupplemented)
E. hirae	29212	Todd Hewitt Broth (unsupplemented)
S. pyogenes	49399	Todd Hewitt Broth (unsupplemented)
S. pneumoniae	6303	Todd Hewitt Broth (unsupplemented)
E. coli	25922	Mueller Hinton Broth (unsupplemented)
P. vulgaris	8427	Mueller Hinton Broth (unsupplemented)
K. pneumoniae	13383	Mueller Hinton Broth (unsupplemented)
P. aeruginosa	25416	Mueller Hinton Broth (unsupplemented)
E. faecalis	11823	Todd Hewitt Broth (unsupplemented)
E. faecium	6569	Todd Hewitt Broth (unsupplemented)

Both ampicillin and tetracycline are used as antibiotic positive controls in each screening assay for *E. coli* (ATCC 25922) and *S. aureus* (ATCC13709).

2.4.3 Cellular Toxicity Testing

The results of this assay can be found in the tables throughout this annual report. The MTT cell proliferation assay is used to test our compounds for cell toxicity. This assay is purchased from American Type Culture Collection (Manassas, VA, USA,) and run according to the manufacturer's protocol (MTT Cell Proliferation Assay; Catalog Number 30-1010k). In summary, the MTT cell proliferation assay measures cell viability and growth by the reduction of tetrazolium salts. The yellow tetrazolium salt is reduced in metabolically active cells to form purple formazan crystals which are solubilized by the addition of detergent. The color is quantified by spectrophotometric means. For each

cell type a linear relationship between cell number and absorbance is established, enabling quantification of changes.

2.4.4 Animal Toxicity Testing

Prior to evaluation in the mouse protection model as described in our protocol, the route of administration and maximum tolerated dose for our compounds are evaulated. This toxicity study provides initial information on toxicity which is required in the design of dose-ranging efficacy studies. Four compounds had advanced for initial animal toxicity testing, **389**, **390**, **392** and **393**.

Compounds **392** and **393**: A single dose of substance (150, 75 and 37 milligrams per kilogram) was administered to a group of 5 and 3 balb-c mice, respectively, by subcutaneous route. The animals were observed for survival for one week. All animals survived at all doses. Furthermore, a histopathological examination of tissues showed no abnormalities, indicating the compounds have little or no acute toxicity.

Compounds **389** and **390**: Initially it was observed that insolubility was limiting our ability to dose the urea analogs. Therefore compound **389** was reformulated as a lactate salt and compound **390** was formulated as its hydrochloride salt. Both reformulations proved to be helpful in increasing solubility. Similar to the experiment described above, a single dose of substance (75, 37, 18 and 9 milligrams per kilogram) was administered to a group of 5 balb-c mice, individually, by interpareteneal (IP) route. At the highest dose, 0/5 mice remained alive in the compound **389** treated group while 3/5 mice were alive in the compound **390** treated group. (After four days one more mice died, and after 11 days only one mice remained alive). At 37 mg/kg, 0/5 mice remained alive in the compound **389** treated group while all mice were alive in the compound **390** treated group. At the 18mg/kg and 9mg/kg dose, all mice survived. These results indicate that compound **389** is more toxic than compound **390**, however, we felt the therapeutic window may be broad enough to accommodate the moderate toxicity of compound **389** and the minimal toxicity of compound **390**.

2.4.5 Animal Models of Antimicrobial Activity

Potential antimicrobial agents are initially evaluated in a low stringency $1xLD_{90}$ test. Mice are inoculated via the injection route with a $1xLD_{90}$ dose of the pathogenic microorganism diluted in hog gastric mucin. Mice are treated with antimicrobial agent immediately after infection. Death of infected, untreated animals generally occurs within 24 to 48 hours. Samples of blood from animals that die are plated on agar plates to confirm infection. Animals are observed for one week and the number of survivors recorded. The 50% protective dose (PD_{50}) is calculated in mg/kg. While the PD_{50} values of known antibiotics do not always correlate with the doses of these agents employed clinically, there is an overall correlation between activity in the mouse model and clinical effectiveness. Potential antimicrobial agents showing activity in the $1xLD_{90}$ mouse protection assay will be screened in a more stringent test ($10xLD_{50}$) and in more complex antimicrobial animal models (e.g. rabbit endocarditis or pneumonia in mice) that closely resemble specific human infections and yield information on potential therapeutic effects in humans.

Six protection studies were carried out according to the guidelines we had outlined in our previous proposal For these experiments we implemented the four analogs (i.e. compounds **389, 390 392** and **393,** *vida supra)* which had passed all levels of our workflow and had advanced to the animal protection experimental stage. For compounds **389** and **390**, we had found that S. aureus was the strain possessing the greatest activity. A standard mouse protection assay was implemented as originally outlined. However, for compound **392** and **393**, the preferred target was found to be Enterococcus. The more complex, mouse thigh model, was chosen rather than the mouse protection assay This was done because; 1) It is in difficult to sytemically infect animals with Enterococcus and the thigh model has been the generally accepted method for testing drugs against such strains *in vivo*, and 2) The thigh model is a more complex model; the drug is injected (nape of the neck) at a site distant from the site of infection (thigh) and therefore, the drug must posses pharmacokinetic properties sufficient to elicit reduction in bacteria. Herein we outline the results of those experiments.

Mouse Thigh Model Protection Experiments (Compounds **392** and **393**): This model is used to measure reduction of Bacterial Burden as well as give an indication that the compounds show Tissue Pharmacokinetics. Mice strain SCID -ICR 4 weeks old were treated Day -4 with 150 mg/Kg cyclophosphamide IP and on Day -1 with 100 mg/kg Cyclophosphamide IP. The mice were treated with Probonecid 1 mg/kg three days prior to compound treatment to induce reduced transient kidney secretion. The right thigh of the mouse is inoculated with 106 Enterococcus faecalis ATCC Clinical Isolate 29212 in 0.1 ml PBS. The mice were treated with compound at appropriate concentrations Sub/Q 2 hr later and were then sacked at 24 hr. The thighs were removed stripped of fat and homogenized manually. The homogenate was serially diluted 10 fold and plated on Tryptic Soy agar.10 control mice (non treated were used to determine the Bacterial concentration in the thigh 24 hr post infection. the compounds used were compound **392** and compound **393**. Vancomycin was the control Drug.

It can be observed from chart 1, that ***both compounds were able to get to the tissue site and were able to reduce the bacterial counts from the controls in a dose dependent fashion.*** Interestingly, compound **392** was more potent than compound **393**. Unfortunately, analysis of kidney and liver sections after necropsy indicates severe toxicity which severely limits these compounds as therapeutics.

Chart 1. Study Summary

Average Bacterial counts for 10 control Mice was $3.9x10^8$ /thigh
All other groups had 5 animals
Bacterial Counts of Vancomycin 1 mg/Kg Treated 0/ thigh

Compound **392** 150 mg/kg Average counts per 5 mice $18.9x10^6$ Represents a 96% reduction in colony count from the control
Compound **392** 75 mg/kg Average counts $156x10^6$ per thigh a 60% reduction
Compound **392** 37 mg/Kg Average counts $225x10^6$ per thigh a 42.3% reduction

Compound **393** 150 mg/kg Average Counts 68.4x106 an 82.5% reduction
Compound **393** 75 mg/kg Average Counts 186.3 $x10^6$ a 36% reduction

Compound **393** 37 mg/Kg Average Counts 102 x10^6 counts greater than control no reduction

Mouse Protection Experiment, Initial dosing (Compounds **389** and **390**):

Compound **390,** the Urea HCl salt was dosed at 3hr and 9hr post Infection with75mg/kg down to 4 mg/kg 0.1 ml IP. Compound **389**, the Urea Lactate Salt, was dosed at 3hr and 9hr post Infection 37mg/kg down to 4 mg/kg 0.1 ml IP. The control, Vancomycin, was dosed at 3hr and 9hr post Infection 1mg/kg and 100ug/kg 0.1 ml IP.

Mouse Strain: Balb/c females 4 weeks of age

Staph. aureus ATCC13709 was grown overnight at 37°C OD 2.0 Concentration: 3x10^9/ml. The Staph was then diluted in PBS, serial tenfold dilutions.
To increase virulence, the Staph was mixed with 5% Hog Gastric Mucin. And Balb/c females 4 weeks of age were challenged with the bacteria.

Chart 2. Summary of Mouse Protection Study.

Group I: 5 mice challenged IP with 10^9/ml Staph. 0.5 ml IP, 5/5 mice dead

Group II: 5 mice challenged with 10^6/ml Staph . 0.5 ml IP, 5/5 mice dead

Group III: 10 mice challenged with 10^6/ml Staph in 5% Hog Gastric Mucin, 9/10 mice dead

Group IV: 5 mice 5% Hog Gastric Mucin 0.5ml IP, 0/5 dead

Group V: HCl Salt Treatment: All mice infected with 10^6/ml Staph in 5% Hog Gastric Mucin

75 mg/kg, 9/10 dead
37mg/kg, 9/10 dead
19 mg/kg, 9/10 dead
9 mg/kg, 9/10 dead
4 mg.kg, 9/10 dead

GroupVI: Lactate Salt Treatment All mice infected with 10^6/ml Staph in 5% Hog Gastric Mucin

37 mg/kg, 10/10 dead
19 mg/kg, 10/10 dead
9 mg.kg, 9/10 dead
4 mg/kg, 9/10 dead

Group VII: Vancomycin Treated Group Vancomycin was diluted in Cremaphor same as Test Compounds All mice infected with 10^6/ml Staph in 5% Hog Gastric Mucin

1 mg/kg 0.5 ml IP, 0/10 dead
100ug/kg o.5 ml IP, 0/10 dead

Mouse Protection Experiment, Modified dosing (Compounds **389** and **390**): Compound **390,** the Urea HCl salt was dosed at 1hr and 3hr post Infection with75mg/kg down to 4 mg/kg 0.1 ml IP. Compound **389**, the Urea Lactate Salt, was dosed at 1hr and 3hr post Infection 37mg/kg down to 4 mg/kg 0.1 ml IP. The control, Vancomycin, was dosed at 1hr and 3hr post Infection 1mg/kg and 100ug/kg 0.1 ml IP. Test animals were inoculated with 10^6 Staph. aureus in 7.5% sterile Hog gastric mucin in DPBS 0.5 ml IP.

Chart 3. Summary of Mouse Protection Study (Modified Dosing)

Group	Dose	Total Dose	Number Alive /Total Number
I	10^8 Staph no mucin	N/A	0/5
II	10^6 Staph no mucin	N/A	5/5
III	10^6 Staph 7.5% mucin	N/A	0/10
IV	Mucin Only	N/A	5/5
V	75 mg/Kg in 0.1 ml (compound **390**)	150 mg/kg	4/10
VI	37.5 mg/Kg in 0.1 ml (compound **390**)	75 mg/Kg	7/10
VII	18.8 mg/Kg in 0.1 ml (compound **390**)	37 mg/Kg	6/10
VIII	9.4 mg/Kg in 0.1 ml (compound **390**)	18 mg/kg	6/10
IX	4.7 mg/Kg in 0.1 ml (compound **390**)	9 mg/Kg	4/10
X	2.3 mg/Kg in 0.1 ml (compound **390**)	4 mg/Kg	3/10
XI	37 mg/Kg in 0.1 ml (compound **389**)	75 mg/kg	3/10
XII	18.8 mg/Kg in 0.1 ml (compound **389**)	37 mg/kg	3/10
XIII	9.4 mg/Kg in 0.1 ml (compound **389**)	18. mg/Kg	4/10
XIV	4.7 mg/Kg in 0.1 ml (compound **389**)	9 mg/Kg	2/10
XV	2.3 mg/Kg in 0.1 ml (compound **389**)	4 mg/KG	0/10
Xvl	1 mg/Kg in 0.1 ml Vancomycin	2 mg/kg	5/5

As can be seen from Chart 3, the results were quite encouraging. For compound **390** (HCl Salt) and **389** (Lactate Salt) a dose dependent increase in survival rate can be observed. However, as the dose increases above 18 mg/kg for compound **389**, or above 75 mg/kg for compound **390**, a decrease in survival rate can be observed, indicating that we are passing through the therapeutic window and reaching toxic levels of drug. Moreover, analysis of the kidney and liver after necrospy indicated a severe phenotype which suggests that further development of these compounds, per se, would likely be unwarranted.

KEY RESEARCH ACCOMPLISHMENTS

- Several hundred analogs of gougerotin were prepared and evaluated in both the coupled transcription/translation assay and bacterial growth-inhibition assays.

- Acyclic Gougerotin Chemistry has been developed and several key analogs are currently being prepared and tested in due course.

- A 20 fold improvement was obtained in inhibition of Prokaryotic transcription/translation compared to the parent, gougerotin.

- A 8-20 fold improvement in bacterial-growth inhibition against *E. Coli* was achieved compared to the parent gougerotin; the best compounds contain an amino-group on the terminus of the first peptidic residue at N-4', a piperazine at the 6'-carboxyl substituent, and an aromatic group at N-6.

- Several hundred biaryl guanidines were prepared and evaluated in both the coupled transcription/translation assay and bacterial growth-inhibition assays.

- The chloro-guanidines were discovered to be weak (50–100 μM, 15–30 μg/mL) inhibitors of gram-positive and gram-negative pathogenic bacteria.

- Design, synthesis and biological testing of conformationally strained chloro-guanidines led to the discovery of the several dichlorobenzimidazoles which showed activity against a range of pathogenic bacteria (2-10 μM, 1–5 μg/mL). This activity is sufficient to warrant sending these compounds out for further testing.

- A vast array of urea and thioureas were prepared and evaluated in both the coupled transcription/translation assay and bacterial growth-inhibition assays.

- Two ureas were discovered to be good inhibitors of gram-positive and gram-negative pathogenic bacteria.

- Several Mouse Protection Assays on two compounds in the urea class showed no protection under one dosing regimen, and good protection under another. This is the first example of this class showing ***a demonstrated ability to protect balb-c mice against S. aureus pathogenic bacteria,*** which warrants additional testing in more advanced models.

- A Mouse Thigh Model of protection was run against two members of the benzimidzaole class. In this experiment we ***demonstrated a dose dependent reduction in pathogenic Enterococcus in balb-c mice*** which warrants additional testing in more advanced models.

3 REPORTABLE OUTCOMES

One manuscript has been submitted and three have been published:

1. "The Solid-Phase Total Synthesis of Gougerotin and Related Analogs", *Organic Letters*, **2004, in review**.
2. "Aryl urea analogs with broad-spectrum antibacterial activity", *Bioorganic & Medicinal Chemistry Letters* **2004,** 14, 5569-5572.
3. "Synthesis and biological evaluations of novel benzimidazoles as potential antibacterial agents." *Bioorganic & Medicinal Chemistry Letters* **2004,** 14, (5), 1217-1220.
4. "2-Piperidin-4-yl-benzimidazoles with broad spectrum antibacterial activities." *Bioorganic & Medicinal Chemistry Letters* **2003,** 13, (19), 3253-3256.

Drafts of these papers are included in the appendix. Other papers detailing this work will be submitted in due course.

4 CONCLUSIONS

A significant degree of completion of all of our ouriginal tasks as outlined in the proposal for award DAMD17-02-2-0023 has been accomplished. Task 1. We have achieved a 20 fold improvement in inhibiting prokaryotic transcription/translation compared to our parent, gougerotin. Also, and 8-20 fold improvement in bacterial-growth inhibition against *E. Coli* was achieved compared to the parent gougerotin. These results have suggested a direction towards the design and synthesis of acyclic analogs of the pyranosyl cytosines that have a high degree of potential to overcome the disparity between the excellent transcription/translation inhibition properties and the less superlative antimicrobial properties. We have synthetic methodology for a series of acyclic analogs and have prepared these analogs. Unfortunately, activity sufficient to advance the pyranosyl cytosines was not present. Task 2-4. We have prepared and evaluated a vast array of guanidine analogs and have, likewise, developed a large SAR dataset from which we identified some dichloroguanidines as having modest antimicrobial activities against gram-positive and gram-negative pathogenic bacteria. Further modification that involved constraining the ring and tethering an amino side chain to the resultant benzimidazole gave us a series of compounds with good antibacterial activity against several strains. The activity observed was significant and has prompted us to initial toxicity testing in balb-c mice and found these compounds to have a LD_{50} well above 140 mg/kg. Subsequent evaluation in a mouse thigh model of infection against pathogenic Enterococcus showed significant. Additionally, the evaluation of urea and thiourea analogs led to the discovery of two urea analogs (one additional urea proved too toxic in cellular assays) that were tested in animal models of toxicity. They both displayed moderate to mild toxicities. Further testing in animal models of protection (S. aureus) led to the demonstration of protection against pathogenic S. aureus bacteria. Unfortunately, examination of the kidney and liver after necropsy indicates that these compounds are unsuitable for further development.

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The Solid-Phase Total Synthesis of Gougerotin and Related Analogs

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Natural products that target RNA include; the macrolides (e.g. erythromycin, clarythromycin, azithromycin),[1] and the amino glycosides (e.g. tobramycin, gentamicin)[2-4]. Each of these has a remarkable ability to efficiently inhibit protein synthesis, resulting in excellent antibacterial activity, and has been the subject of many reports. However, the hexopyranosyl cytosines, a large class of RNA binding natural products, has been less well explored. Members of this class are structurally related containing a cytidine base attached to a 6-membered ring sugar/s and often a modified peptidic moiety. Compounds in this class include; bagougeramine A, blasticidin S, and gougerotin (Figure 1).[5-7] Experiments have suggested a common binding site for each compound which is located near the peptidal transferase region of the large subunit of rRNA.[8,9] Common points of contact of these compounds with the structured rRNA has been previously noted.[10]

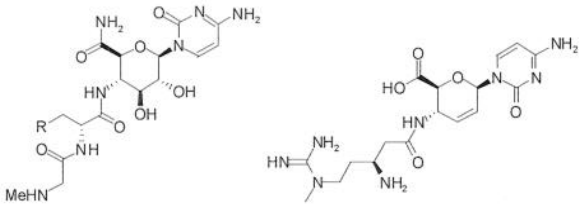


Figure 1. Several Hexopyranosyl Cytosines.

The most frequently studied hexopyranosyl cytosine, gougerotin, has been obtained either by isolation from the fermentation broth of *Streptomyces gougeroti*[11] or through total synthesis[12,13]. No semi-synthetic analogs have been reported in the literature, and no more than thirty analogs based on the total synthesis have been reported. [7,14-16] Additionally, compounds with improved activity relative to the parent compound, have not been identified, and literature reports of additional synthetic work on gougerotin have remained absent since the 1970s. As part of our program to discover novel classes of compounds that bind RNA, we were intrigued by the fact that a synthesis of gougerotin appeared to be compatible with current solid phase methodology, which would overcome the previous obstacle to advanced structure-activity-relationship studies; i.e. the solution phase synthesis is too complex to be practical for the preparation of a large number of analogs for biological studies. A successful adaptation of the synthesis of gougerotin to solid-phase would allow us unprecedented access into a vast array of analogs and we could rapidly evaluate the hexopyranosyl cytosine class for biological activity.

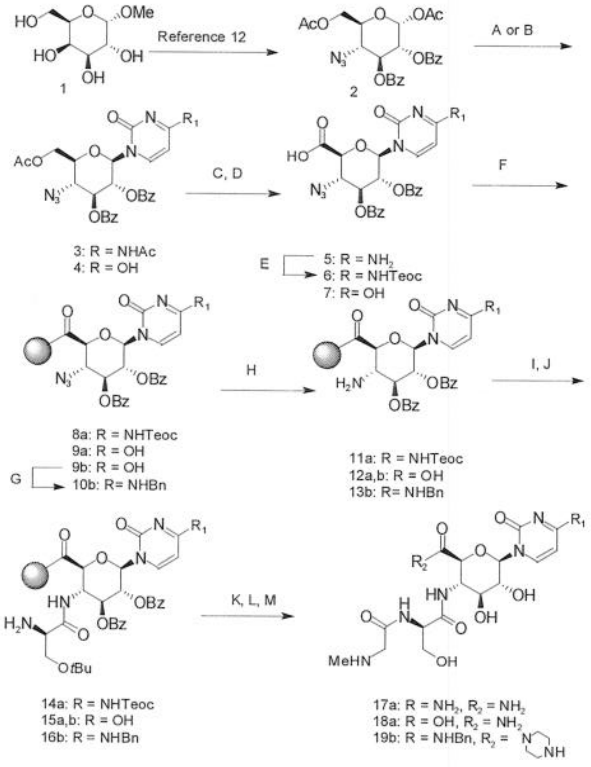
Therefore, we initiated studies towards a solid phase total synthesis of gougerotin that would not only be amenable to high-throughput analog production, but would allow us to access previously unexplored positions; (e.g. N-4, C-5).

In light of the previous difficulties in obtaining analogs synthetically, our initial efforts were directed towards the total synthesis of gougerotin with a focus on key steps initially developed by Fox and Watanabe[12,16-24]. Using literature methods (Scheme 1), we were able to prepare the starting glycosyl donor **2** from commercially available galactoside **1** on a multi-gram basis. This compound was then coupled to give **3** and then deacetylated under the conditions developed by Fox and Watanabe. A one-step oxidation using TEMPO/BIAB[25] was then carried out in CH_3CN (aq) to give the carboxylic acid **5**, in good yield. Protection of the N-4 exocyclic amino group was accomplished to give the Teoc-protected[26] precursor **6**. Our precursor was the coupled with HATU to ArgoGel Rink resin to give the resin-bound precursor **8a**. Reduction of the azido group with Sn(II)chloride liberated the 4'-amino group to give amine **11a**. A subsequent HATU coupling with OtBu-Fmoc-D-Serine followed by Fmoc removal with 10% piperidine/DMF gave amine **14a**. A second HATU mediated coupling with Boc-sarcosine gave the fully protected resin-bound gougerotin. Compound **14a** was treated sequentially with 0.4M NaOH/MeOH (1:5) to remove the benzoates and Teoc carbamate,[27] and then TFA to give gougerotin (**17a**) as its TFA salt. Both 1- and 2-D, ^{1}H NMR and ^{13}C NMR, and HRMS was used to confirm the structure and purity (>90%) of gougerotin.

Next, we coupled uracil to our glycosyl donor **2** under modified Vorbruggen conditions[28] using 3 equivalents of TMS-OTf under refluxing conditions to give an 80% yield of compound **4**. In contrast, the $SnCl_4$ conditions gave only a small amount of product. After deacteylation and oxidation using the conditions developed for the protected cytidine, we were able to obtain our precursor **7**. A HATU mediated coupling then gave our resin-bound uracil derivative **9a.**

Nucleophillic displacement of an appropriately installed leaving group on N-4 was then envisaged as a route to those analogs. Treatment of uracil **9a** with a preformed triazolating reagent[29] (i.e. 1,2,4-triazole, $POCl_3$ and Et_3N in CH_3CN) for 4 hours followed by nucleophilic aromatic substitution with $BnNH_2$ failed to give any desired product. This was, however, believed to be due to either the primary amide or homogeneous nature of the reagent, as this reaction has never been reported on solid-phase. Therefore, compound **7** was coupled to ArgoGel-MBCHO[10] resin, functionalized as a piperazine, to give

compound **9b**. Subjecting this compound with the preformed triazolating reagent followed by treatment with benzylamine in ethanol gave the putative intermediate **10b**. A small amount of this resin was then deprotected with TFA to give the desired product (LCMS [M+H]= 679.2 *m/z*, >95% purity). The remainder of the sequence was then affected (coupling both fragments and deprotection) to give the N-4 substituted gougerotin precursors **19b**. A similar sequence was carried out on the unfunctionalized uracil derivative **9a** and **9b** to give the uracil derivatives of gougerotin, **18a** and **18b**, respectively. A similar sequence was carried out on compound **6** using the piperazine-resin to give compound **17b**. In all cases, purities of 85 to >95% were achieved after cleavage from the resin, as determined by LC/MS, and these compounds could be assesed for biological activity without the need for any further purification.



[a] A) N-Acetylcytidine, $SnCl_4$, DCE, 80°C, 24h ; B) Uracil, TMS-OTf, CH_3CN, 70°C, 48h; C) 2 equiv. Et_3N, MeOH, 16h; D) TEMPO (cat.), BIAB, 80% CH_3CN (aq.), 25°C, 4h; E) Teoc-OSu, DIPEA, DMF, 60°C, 12h; F) Resin (see below), HATU, DIPEA, DMF, 25°C, 48h; G) 1,2,4-triazole, Et_3N, CH_3CN, $POCl_3$, 25°C, 4h then 10% $BnNH_2$/EtOH, 25°C, 10h; H) $SnCl_2$, PhSH, Et_3N, DCM, 25°C, 1h; I) Ot-Bu-FMOC-D-Serine, HATU, collidine, DMF, 25°C, 16h; J) 10%piperidine/DMF, 25°C, 1h; K) Boc-sarcosine, HATU, collidine, DMF, 25°C, 16h; L) 0.4M NaOH/MeOH (1:4), 25°C, 5h; M) TFA, 25°C, 4h.

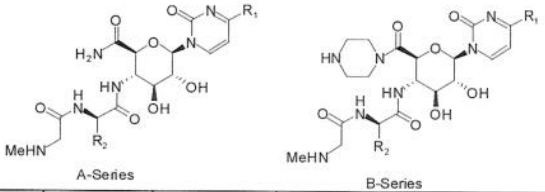
[b] a = ArgoGel-NH_2, b = ArgoGel-

Scheme1. Gougerotin Synthesis.

A small test library of 12 compounds was prepared and screened for biological activity (Table 1) by appropriate substitutions of the procedure outlined in Scheme 1 (i.e. D-serine to D-diaminobutyric acid, benzylamine to phenethylamine). The results were very encouraging. Small changes in structure were found to have a dramatic impact on the activity of inhibition of peptide bond formation (i.e.inhibition of transcription/translation, T/T) and against pathogenic *E. Coli*.and *C. Albicans*. The presence of a nitrogen at position-4 was absolutely required for activity. Additionally, substitutions at the amide portion of the sugar (series B), the presence of an alkylaryl group on N-4 , and the D-diaminobutyric acid substitution at the first peptidic moiety all had positive effects on activity. For example, compound **22b** showed an approximate 4-fold increase over the parent against pathogenic *E. Coli* (the MIC for gougerotin is >200uM). Most interestingly, compound **20b** showed <3uM MIC against *C. Alibicans*, which demonstrates a potential new class of antifungal therapeutic.

In conclusion, we have develolped a solid-phase synthesis of the natural product gougerotin, and have prepared a small test library demonstrating this methodology. Positions amenable to substitution include; N-4, the 6'-amide, the first and second peptide residues, and should be amenable to substitutions at C-5 and C-6. The purity of our analogs allows us to assess our compounds for biological activity without the need for chromatography or other purification. Most encouraging though, is the new and improved bilogical activities discovered using only a very small subset of the potential analogs this methodology makes available. We are currently seeking to prepare several hundred analogs and further expand out methodology.

Table 1. Functionalized Gougerotin Analogs.



Cmpd	R_1	R_2	Series	T/T[a] IC_{50}, (μM)	MIC[b] *E. Coli* (μM)	MIC[b] *C. Albicans* (μM)
17a	NH_2	CH_2OH	A	0.5	>200	50-100
18a	OH	CH_2OH	A	>100	>100	>100
20a	NH_2	$CH_2CH_2NH_2$	A	0.2	>100	>100
21a	OH	$CH_2CH_2NH_2$	A	>100	>100	>100
17b	NH_2	CH_2OH	B	0.4	>100	>100
18b	OH	CH_2OH	B	>100	>100	>100
19b	NHBn	CH_2OH	B	0.5	>100	>100
20b	NH_2	$CH_2CH_2NH_2$	B	0.3	50-100	1-3
21b	OH	$CH_2CH_2NH_2$	B	>100	>100	>100
22b	$NHCH_2Bn$	$CH_2CH_2NH_2$	B	0.5	25-50	nt[c]
23b	$NHCH_2Bn$	CH_2OH	B	2.3	>100	nt[c]
24b	NHBn	$CH_2CH_2NH_2$	B	0.4	50-100	6-12

[a] T/T = Inhibition of the transcription/translation sequence in procaryotic system.
[b] Minimum Inhibitory Concentration
[c] Not Tested

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SUPPORTING INFORMATION AVAILABLE Experimental procedures, and spectral data for all compounds (PDF). This material is available free of charge via the internet at http://pubs.acs.org.

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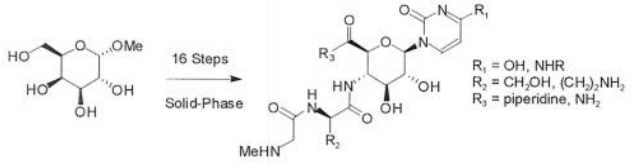
Abstract:



Gougerotin: R_1=R_3 = NH_2, R_2 = CH_2OH

The first solid phase synthesis of the natural product, Gougerotin, has been accomplished. The synthetic route is versatile and allows for diversification at position C-4 of the heterocycle, C-6' of the sugar ring and both residues of the peptidic moiety at N-4' in a parallel fashion.

The Solid-Phase Total Synthesis of Gougerotin and Related Analogs

Supporting Information

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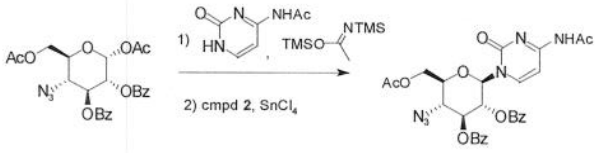
Index

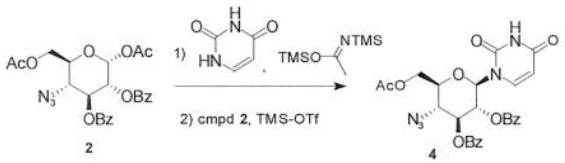
1. Solution-Phase Synthesis of Resin Precursors.....pp. S1-3
2. Solid-Phase Synthesis.....pp. S4-5
3. Library Data and Gougerotin Spectral Data.....pp. S6-11
4. Biology Experimental Section.....pp. S12

1. Solution-Phase Synthesis of Resin Precursors



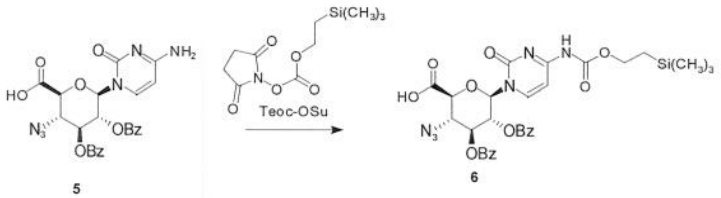
2 3

Protected Nucleoside 3. N-Acetyl cytidine (7.8g, 50.9 mmoles) was suspended in dichloroethane (500 mL) and bis(trimethylsilyl)acetamide (10.5 mL, 60.0 mmoles) was added dropwise. The reaction mixture was then heated at 65°C to affect a clear solution. The clear solution was then cooled to room temperature, at which time the sugar precursor **2** (Toronto Research Chemicals, 14.7 g, 29.5 mmoles) was added in one portion followed by the dropwise addition of tin(IV)chloride (13mL, 111 mmoles). The resultant solution was stirred for 24 hours at room temperature, and then poured into (very slowly!) a saturated $NaHCO_3$ solution (400 mL). The organic layer was separated and washed with water (200 mL), dried (MgSO4), filtered and evaporated to an oil. The oil was then treated with acetone (400 mL) and the resultant solids were collected by filtration and dried under reduced pressure overnight to give 10.7 g (61%) of nucleoside **3** which was used without further purification. ^{1}H NMR (300MHz, DMSO-d_6) δ 10.83 (s, 1H), 8.23 (d, 1H, J = 7.0 Hz), 7.88 (d, 1H, J = 7.0 Hz), 7.7-7.4 (m, 13H), 7.22, (d, 1H, J = 7.0 Hz), 6.42 (d, 1H, J = 9.0 Hz), 6.04 (t, 1H, J = 5.0 Hz), 5.64 (t, 1H, J = 5.0 Hz), 4.7-4.4 (m, 1H), 3.4 (m, 1H), 2.07 (S, 1H), 2.03 (S, 1H); LCMS (~90%) m/z 613.3 (M+Na)

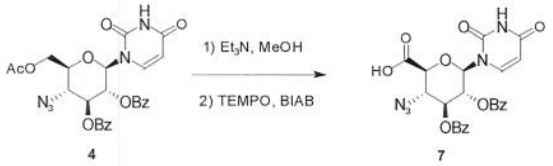


Protected Nucleoside 4. Uracil (2.52 g, 22.5 mmoles) was suspended in CH_3CN (80 mL) and bis(trimethylsilyl)acetamide (6.4 mL, 67.5 mmoles) was added dropwise. The reaction mixture was stirred at room temperature affect a clear solution. The sugar precursor **2** (Toronto Research Chemicals, 7.2 g, 15 mmoles) was added followed by trimethylsilyl trifluoromethanesulfonate (8.1 mL, 101 mmoles) and heated at reflux for two days. The resultant solution was cooled to room temperature and most of the solvent was evapotated. The resultant oil was partitioned between H_2O (100 mL) and EtOAc (200 mL) and the organic layer was washed sucessively with H_2O (100 mL) and brine (100 mL). The organic layer was dried ($MgSO_4$), filtered and evaporated to a syrup, which was purified by column chromatography (2-10% MeOH/CH_2Cl_2). The protected nucleoside was obtained as a white solid (6.7 g, 87%). ^{1}H NMR (300MHz, DMSO-d_6) δ 11.4 (bs, 1H), 7.9-7.4 (m, 11H), 6.26 (d, 1H, J = 8.7 Hz), 6.08 (t, 1H, J = 8.2 Hz), 5.8-5.7 (m, 2H), 4.4-4.1 (m, 3H), 3.4 (m, 1H), 2.06 (s, 3H); LCMS (~90%) m/z 572.0 (M+Na).

Protected Nucleoside 5. Compound **3** (20.4 g, 34.5 mmoles) was added to a mixture of methanol (2 L) and triethylamine (9.5 mL). After stirring two days at room temperature, the solvents were evaporated under reduced pressure and the resultant yellow glass was suspended in Et_2O (500 mL). After stirring overnight, the white solid was collected by filtration, dried under reduced pressure overnight and used without further purification. Total yield was 16.5 g (86%). ^{1}H NMR (300MHz, DMSO-d_6) δ 7.89 (d, 1H, J = 9.2 Hz), 7.8-7.4 (m, 11H), 7.2 and 7.1 (bs, 2H), 6.28 (d, 1H, J = 9.2 Hz), 5.91 (t, 1H, J = 9.4 Hz), 5.73 (d, 1H, J = 7.40), 5.23 (t, 1H, J = 9.2 Hz), 4.98 (t, 1H, J = 5.60), 4.10 (t, 9.8), 3.5-3.7 (m, 2H); ^{13}C (300MHz, DMSO-d_6) 166.0, 165.7, 165.0, 155.4, 134.5, 129.9, 129.8, 129.5, 129.84, 129.82, 129.0, 95.6, 80.39, 77.2, 74.7, 72.0, 61.2, 60.0; (LCMS (>95%) m/z 507.3 (M+H). This intermediate (7.8 g, 15.4 mmoles) was suspended, with vigourous stirring, in a mixture of H_2O (60 mL) and CH_3CN (80 mL) at room temperature for 45 min to affect a smooth white suspension. At that time TEMPO (0.48 mg, 3.1 mmoles) and BIAB (iodobenzene diacetate, 10.4 g, 32.3 mmoles) were added and the mixture was allowed to stir at room temperature for 2 hours and then let stand for 2 additional hours. The solid was collected by filtration, washed with a small amount of acetone (60 mL, cold) and then Et_2O (60 mL). After drying under reduced pressure overnight, 6.6 g (82%) of a white solid was obtained. ^{1}H NMR (300MHz, DMSO-d_6) δ 7.9-7.3 (m, 14H), 6.42 (d, 1H, J = 9.2 Hz), 5.91 (t, 1H, 9.6 Hz), 5.8-5.6 (m, 2H), 4.58 (d, 1H, J = 10.2 Hz), 4.27 (t, 1H, 9.8 Hz); ^{13}C (300MHz, DMSO-d_6) 169.0, 165.9, 165.6, 165.2, 155.1, 142.1, 134.6, 129.9, 129.8, 129.6, 129.5, 129.2, 128.9, 95.9, 80.7, 74.9, 73.7, 71.5, 61.3; LCMS (>95%) m/z 520.2 (M+H).

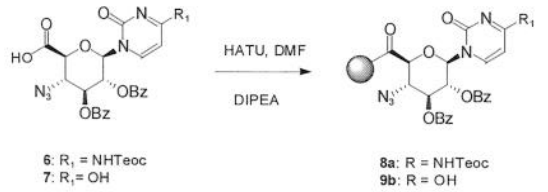


Resin Precursor 6. A mixture of compound **5** (1.8 g, 2.7 mmoles), Teoc-OSu (1.98 g, 7.6 mmoles), diisopropylethylamine (1.62 mL), and CH_3CN (22 mL) was heated at 65°C for 2 days. The reaction was then evaporated to a brown oil, which was dissolved in EtOAc (100 mL), washed with H_2O (50 mL), brine (50 mL), dried ($MgSO_4$), filtered and evaporated to dryness. The resultant oil was triturated with EtOAc/Heaxanes (1:1), filtered and dried under reduced pressure overnight to give 1.7 g (76%) of precursor **6** which was suitable for coupling to resin. ^{1}H NMR (300MHz, DMSO-d_6) δ 10.6 (bs, 1H), 8.30 (d, 1H, J = 12.8 Hz), 7.9 (d, 2H, J = 7.0 Hz), 7.7-7.4 (m, 8H), 7.10 (d, 1H, J = 7.6 Hz), 6.51 (d, 1H, J = 9.0 Hz), 5.96 (t, 1H, 9.60), 5.80 (t, 1H, J = 9.2 Hz), 4.64 (d, 1H, J = 10.2 Hz), 4.3-4.1 (m, 3H), 3.4-3.3 (m, 2H), 1.0-0.9 (m, 2H), 0.0 (s, 9H); LCMS (>95%) m/z 665.2 (M+H).



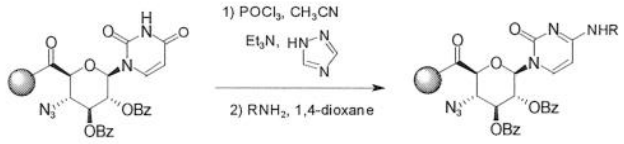
Resin Precursor 7. Compound **4** (2.2 g, 4.0 mmoles) was added to a mixture of methanol (150 mL) and triethylamine (4.0 mL). After stirring one day at room temperature, the solvents were evaporated under reduced pressure and the resultant yellow glass was suspended in hot EtOAc/hexanes (100 mL). After stirring at room temperature for 1 hour, the white solid was collected by filtration, dried under reduced pressure overnight and used without further purification. Total yield was 1.55 g. LCMS (>95%) m/z 530.1 (M+Na). This intermediate (1.55 g, 3.06 mmoles) was suspended, with vigourous stirring, in a mixture of H_2O (15 mL) and CH_3CN (20 mL) at room temperature for 45 min to affect a smooth white suspension. At that time TEMPO (90 mg, 0.6 mmoles) and BIAB (iodobenzene diacetate, 2.1 g, 6.5 mmoles) were added and the mixture was allowed to stir at room temperature for 2 hours and then let stand for 2 additional hours. The solid was collected by filtration, washed with a small amount Et_2O (10 mL). After drying under reduced pressure overnight, 1.2 g (52% over two steps) of compound **7** was obtained as a white solid was obtained. ^{1}H NMR (300MHz, DMSO-d_6) δ 11.4 (bs, 1H), 8.0-7.4 (m, 11H), 6.34 (d, 1H, J = 8.6 Hz), 5.97 (t, 1H, J = 8.4 Hz), 5.8-5.7 (m, 2H), 4.4-4.60 (d, 1H, J = 9.2 Hz), 4.31 (t, 1H, J = 8.8), 3.4-3.3 (m, 1H); LCMS (95%) m/z 522.1 (M+H).

2. Solid-Phase Synthesis

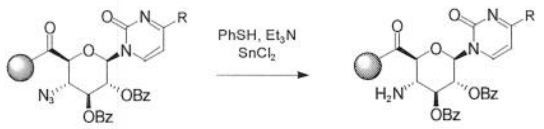


General Procedure for the Coupling of Compound 6 or 7 to Amine Resins. The resin (0.9 mmoles, ArgoGel® Fmoc-Rink* or Piperazine resin) was treated with compound **6** (1.4 g, 2.1 mmoles), HATU (2.1 mmoles) and diisopropylethylamine (900 uL, 5.2 mmoles) in DMF (10mL) overnight. (A Kaiser test can be used to acertain whether a second coupling is required.) The resin was then sequentially washed with (DMF x 5) and (MeOH then DCM x 3). The resin was then dried or carried on directly to the next step. A small portion was cleaved from the resin for testing with 95%TFA-5% triisopropylsilane for 4 hours and then evaporated to dryness. **8a**; LCMS (>95%) m/z 520.2 (M+H). **9a**; LCMS (>95%) m/z 521.1 (M+H), **8b**; LCMS (>95%) 589.1 m/z (M+H). **9b**; LCMS (>95%) m/z 590.2 (M+H).

*Fmoc-Rink was first deproted using 5% piperidine/DMF for 1 hour, followed by washing (DMF x 7).



General Procedure for conversion of uracil to substituted cytidines. A mixture of 1,2,4-triazole (158 mg, 2.3 mmoles) in CH_3CN (2 mL) was treated with the dropwise addition of $POCl_3$ (48 uL, 0.52 mmoles) under nitrogen. The suspension was cooled to 0°C and then Et_3N (320 uL, 2.3 mmoles) was added dropwise. The mixture was stirred at 0°C for 30 min, at which time 0.1 uMoles of resin was added. After stirring for 5 hours, the resin was sequentially washed with (DCM x 5), (DMF x 3) and (MeOH then DCM x 3). After drying the resin for 1hour, 10% of the desired amine in 1,4-dioxane was added and the reaction was allowed to proceed overnight to affect the nucleophilic substitution reaction. The resin was then sequentially washed with (DMF x 5) and (MeOH then DCM x 3). The resin was then dried or carried on directly to the next step. A small portion was cleaved from the resin for testing with 95%TFA-5% triisopropylsilane for 4 hours and then evaporated to dryness. [E.g.**10b**; LCMS (>95%) m/z 679.2 (M+H).]



General Procedure for the Conversion of Azide to Amine. A solution of thiophenol (250 uL, 2.4 mmoles), triethylamine (420 uL, 3.0 mmoles) and tin(II)chloride (114 mg, 0.6 mmoles) in $CDCl_3$ (8mL) was added to 0.3 mmoles of resin. After stirring for 1 hours, the resin was sequentially washed with (MeOH x 5), (DMF x 5) and (MeOH then DCM x 3). The resin was then dried or carried on directly to the next step. A small portion was cleaved from the resin for testing with 95%TFA-5% triisopropylsilane for 4 hours and then evaporated to dryness. [E.g.**13b**; LCMS (>95%) m/z 653.2 (M+H).]

General Procedure for the Coupling of Amino Acids. The resin (0.1 mmoles) was treated with a Fmoc- or Boc-protected amino acid (2.0 mmoles), HATU (2.0 mmoles) and 2,4,6-collidine (5.0 mmoles) in DMF (10 mL) for 4-14 hours. (A Kaiser test can be used to acertain whether a second coupling is required.) The resin was then sequentially washed with (DMF x 5) and (MeOH then DCM x 3). The resin was then dried or carried on directly to the next step

General Procedure for the Deprotection of the Fmoc Group. The resin was covered with a solution of 10% piperidine/DMF for 1 hour, then sequentially washed with (DMF x 5) and (MeOH then DCM x 3). The resin was then dried or carried on directly to the next step. A small portion could be cleaved from the resin for testing with 95%TFA-5% triisopropylsilane.

General Procedure for the Removal of Bz and Teoc Protecting Groups, and Cleavage from the Resin. To a 15uM portion of resin was added a 1 mL solution of 0.4M NaOH/MeOH (1:5) for four hours. The resin was then washed sequentially with (MeOH x 5), (DMF x 5), (AcOH x 3) and (MeOH then DCM x 3). The product was then cleaved from the resin with 95%TFA-5% triisopropylsilane over 4 hours. The solvent was evaporated to dryness, which usually resulted in a hard, syrup. After triturated with CH_3CN, spinning in a centrifuge, decanting the CH_3CN, and drying overnight, the final product could usually be obtained as a white to off-white solid.

3. Library Data and Gougerotin Spectral Data

Gougerotin, our initial test compound, was prepared on a 150 umole scale (80% yield) and 1-D and 2-D spectra were obtained to verify the correct structure. The ^{13}C spectrum matches, almost perfectly, previously published spectra of the naturally occuring gougerotin. In addition, a ^{1}H spectrum, TOCSY and NOSEY spectrum were obtained to confirm strucure. HRMS was 443.1765 calcd. 443.1766 found. LCMS data of gougerotin made on small scale as a member of our library is shown on pages S10-S11.

Figure S1. ^{13}C Spectra of Gougerotin.

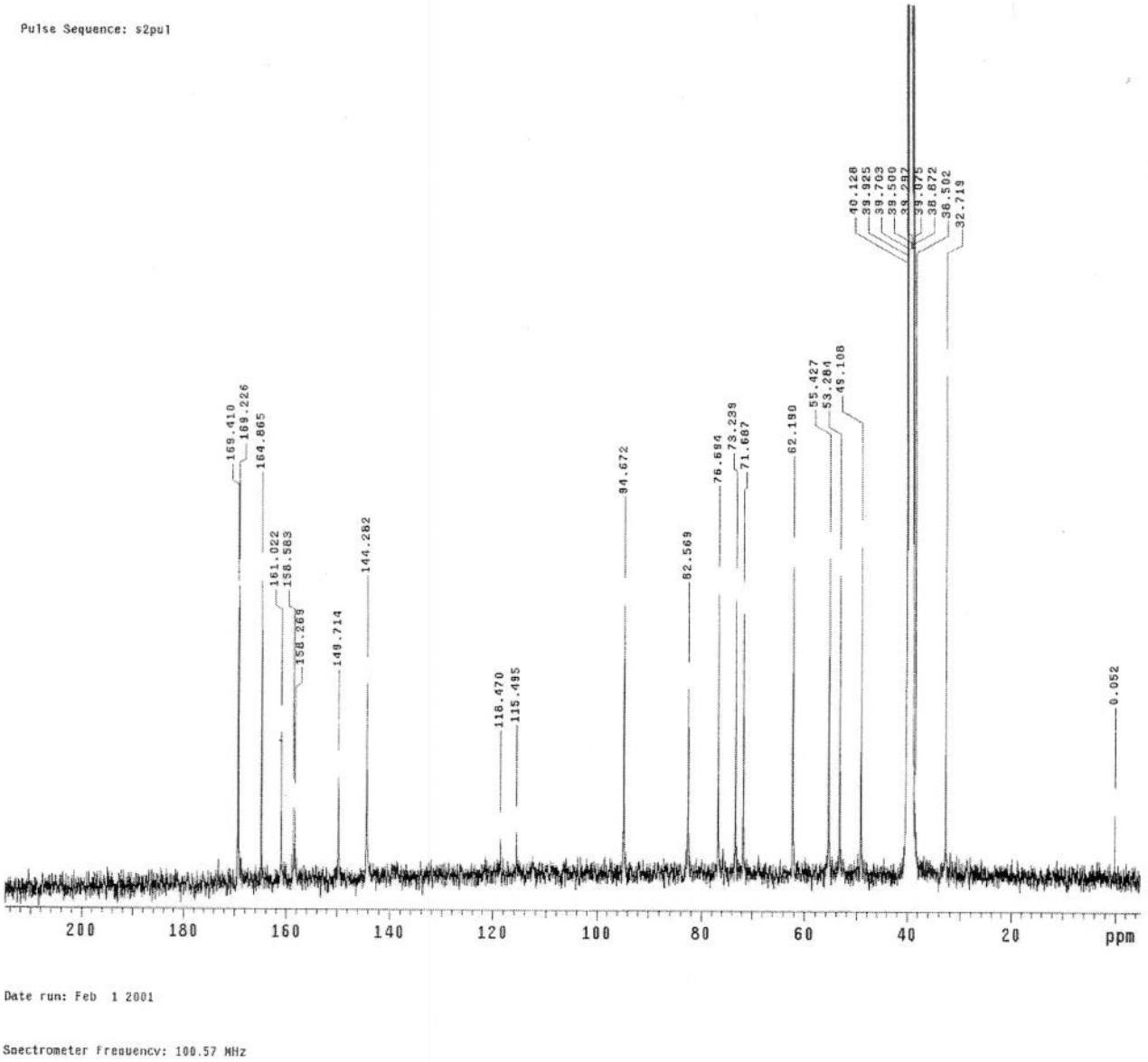


Figure S2. ^{1}H Spectra of Gougerotin.

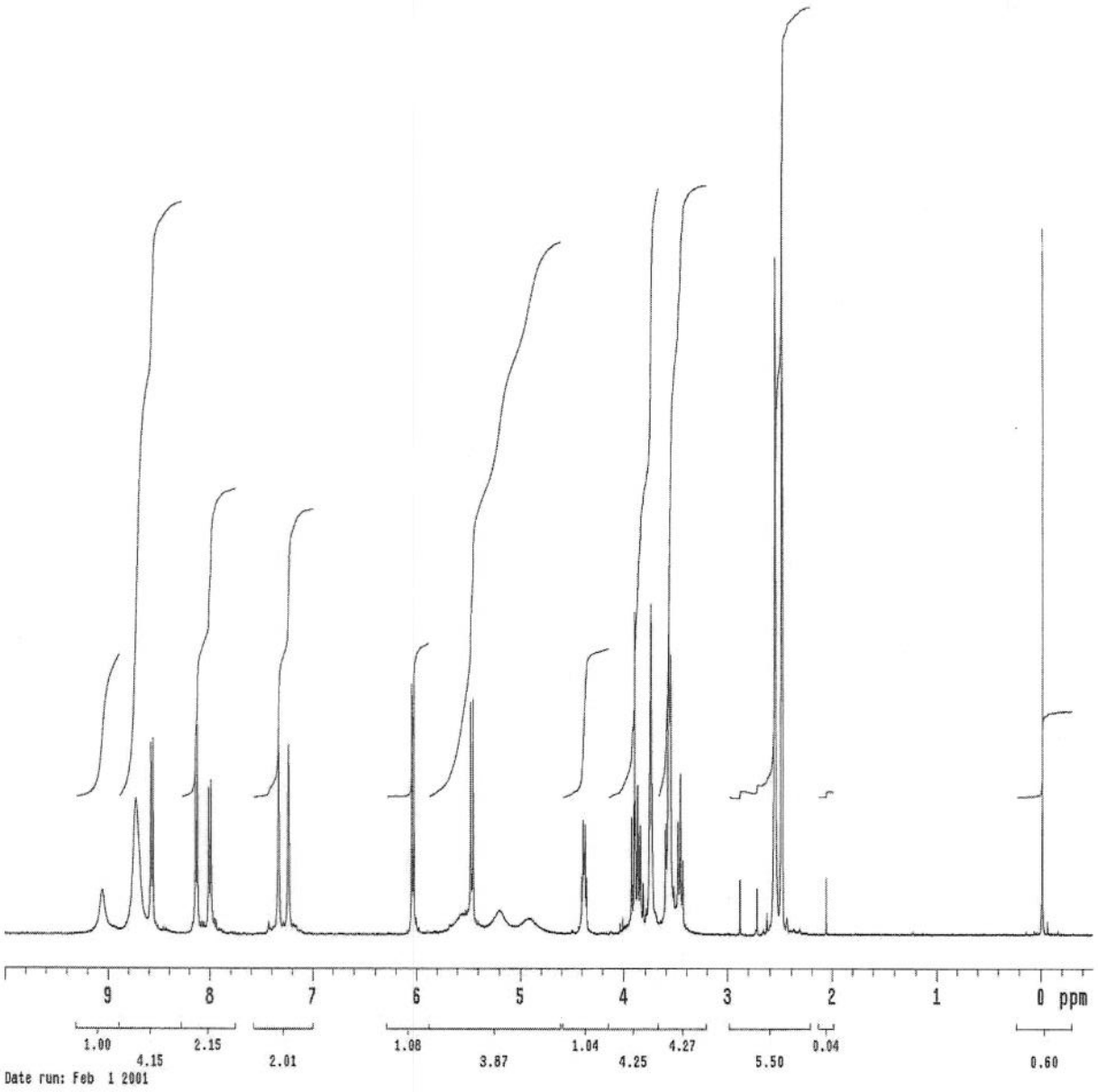


Figure S3. TOCSY ^{1}H-^{1}H Spectra of Gougerotin.

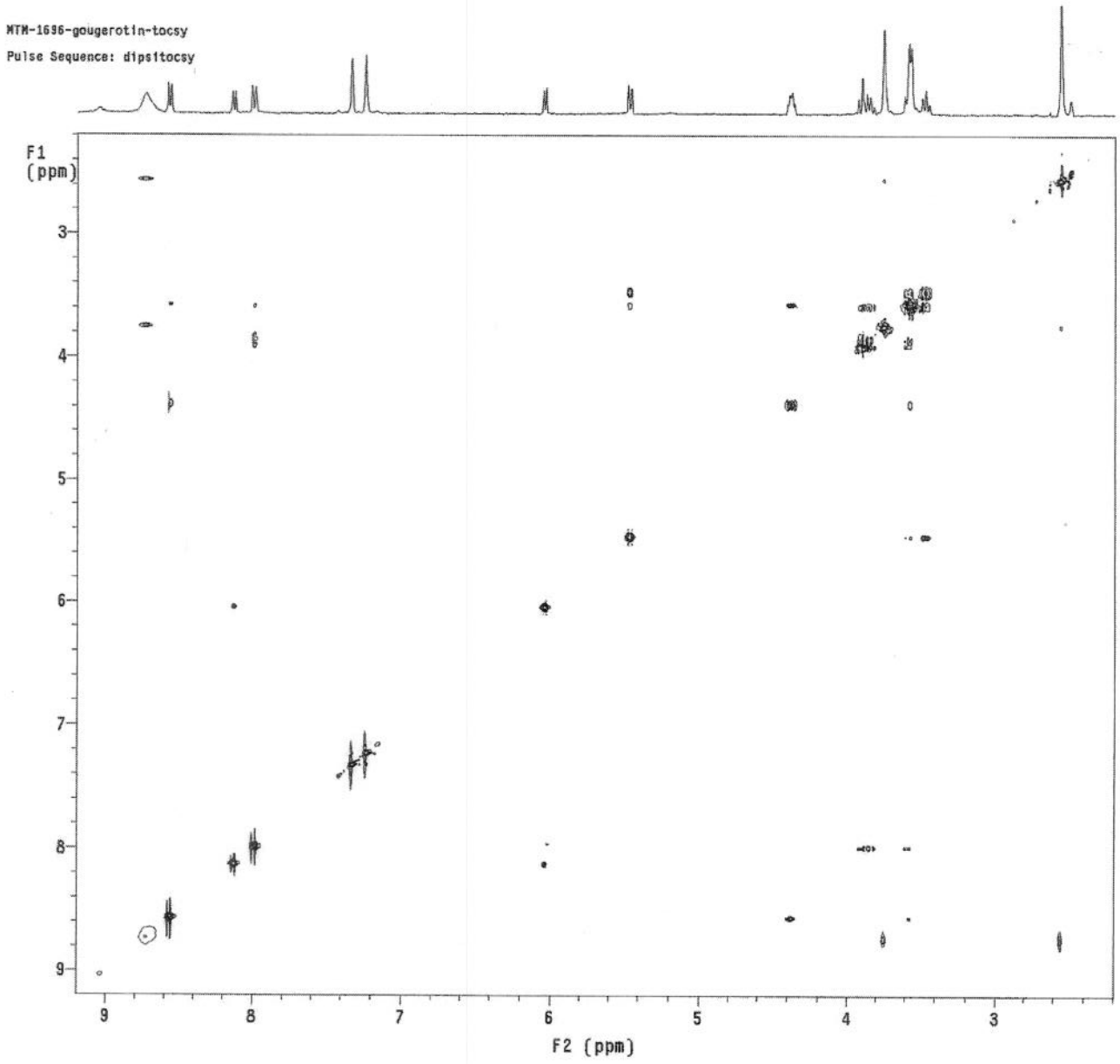
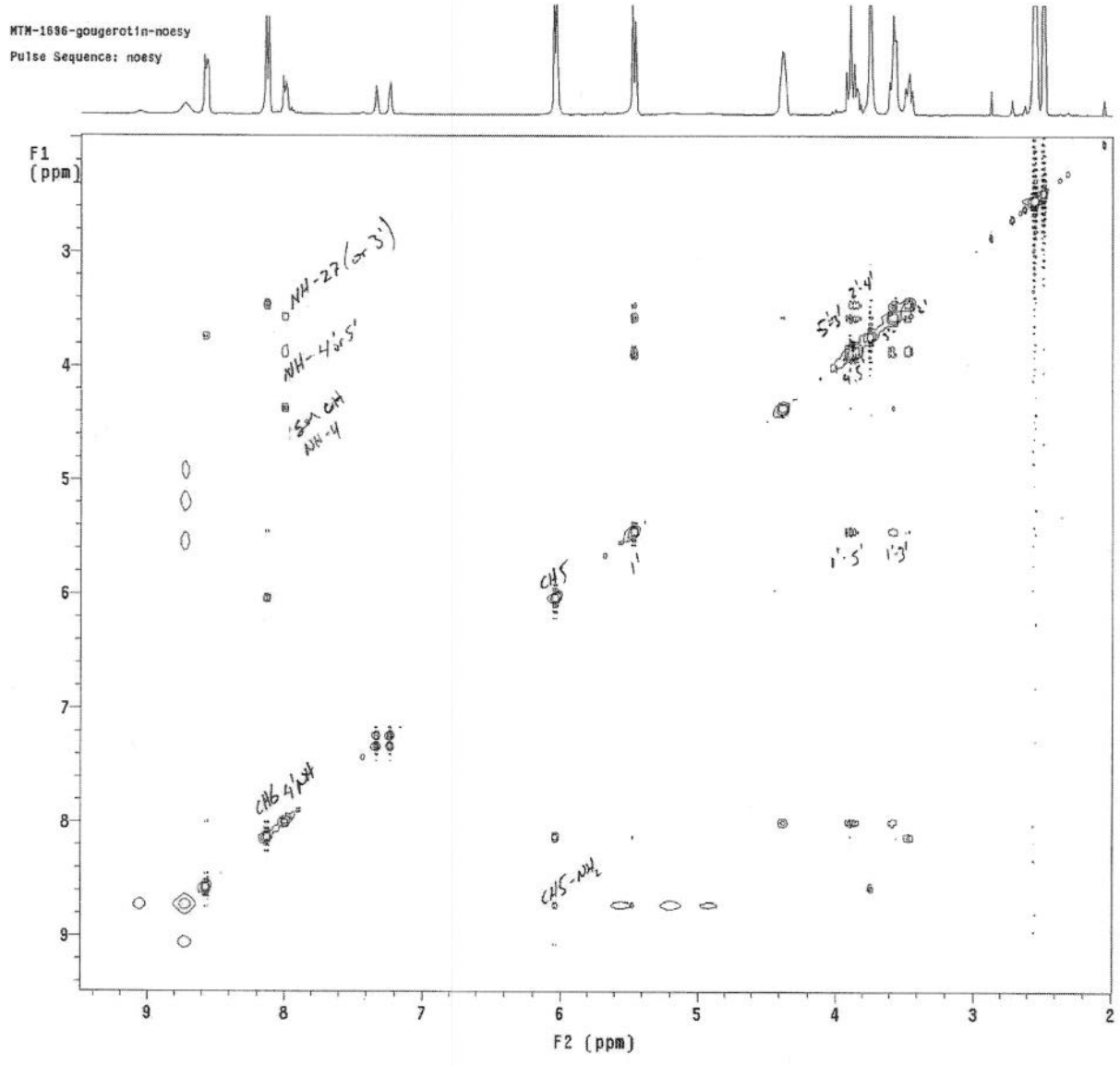


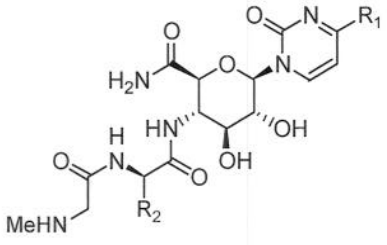
Figure S4. NOSEY Spectra of Gougerotin.



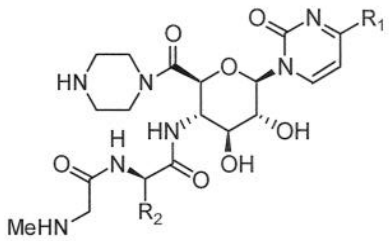
Our libraries are generated, using the procedures from section 2, by substituting for the desired amino acids, amines and reagents. The libraries are run on a Quest 210 in a parallel fashion at a 10-20 umole scale. At the completion of synthesis, we find it useful to obtain LCMS spectra on each compound and sample our library by ^{1}H NMR (300MHz, DMSO-d_6). Usually, the H-1' protons and H-6 protons are clearly discernable, while often others are difficult to assign. The key data is compiled in Table S1 below. ^{13}C Spectra are more useful for structural assignment (as other groups have noted), but are not practical on small scale (<20 umoles).

Table S1. Analytical Data from Library.

All libraries were subjected to LCMS analysis using a 4 x 125 mm 5 uM Lichrosphere Diol column using a gradient of 95% CH_3CN (aq) to 25% CH_3CN (aq) buffered with 10mM NH_4OAc. The eluent was run through a UV cell and then split between an ELSD detector and an Agilent MSD. Purity was judged by integrating the peaks under the ELSD portion of the curve.



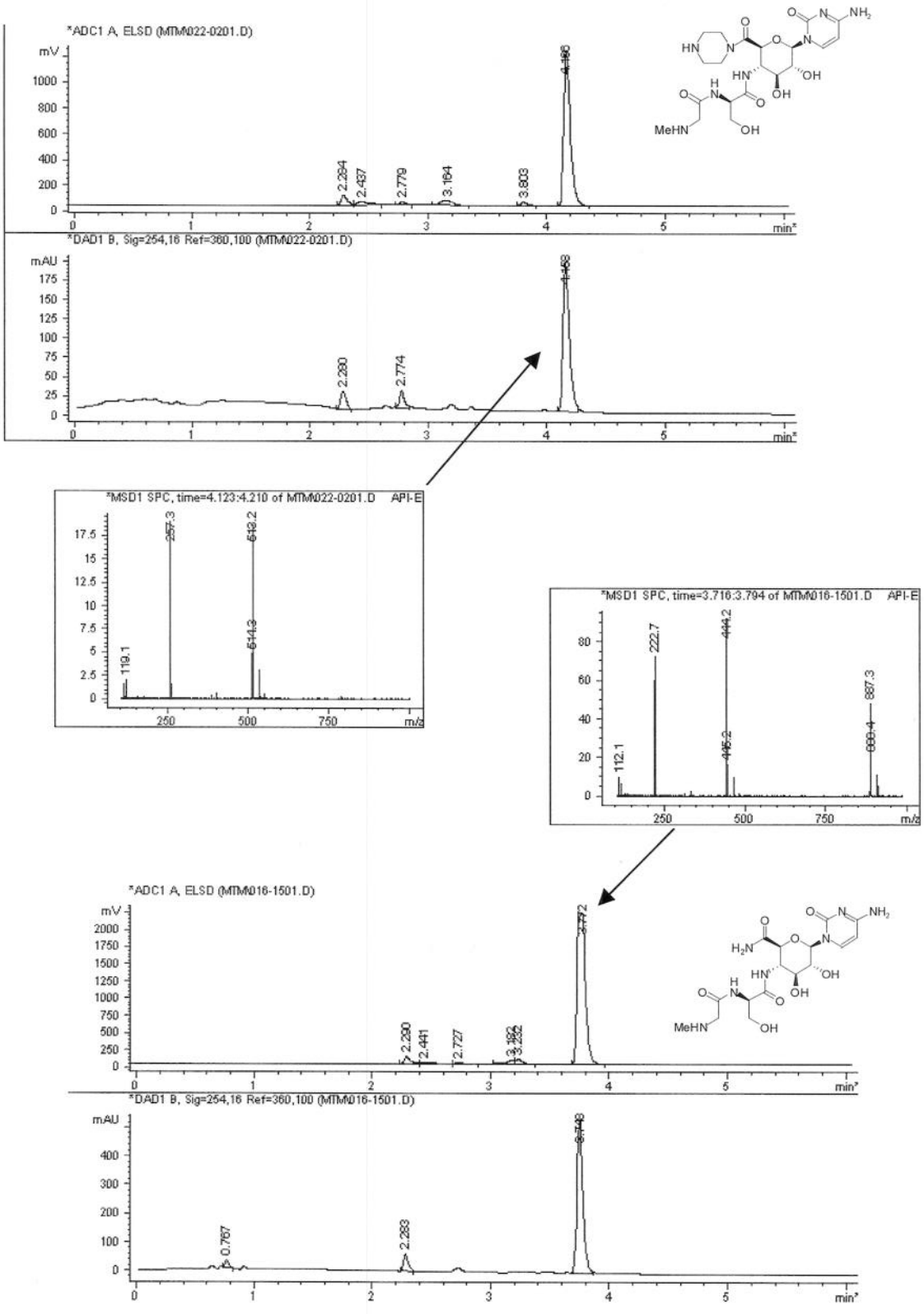
A-Series



B-Series

Cmpd	R_1	R_2	Series	LCMS (m/z)	LCMS Purity (%)	^{1}H NMR (H-6, H-1') (ppm)
17a	NH_2	CH_2OH	A	444.2 (M+H)	95	8.14, 5.47
18a	OH	CH_2OH	A	445.2 (M+H)	86	7.90, 5.40
20a	NH_2	$CH_2CH_2NH_2$	A	457.1 (M+H)	92	8.18, 5.50
21a	OH	$CH_2CH_2NH_2$	A	458.2 (M+H)	91	7.88, 5.40
17b	NH_2	CH_2OH	B	513.2 (M+H)	96	8.02, 5.48
18b	OH	CH_2OH	B	514.2 (M+H)	89	7.81, 5.42
19b	NHBn	CH_2OH	B	603.2 (M+H)	90	8.14, 5.48
20b	NH_2	$CH_2CH_2NH_2$	B	526.3 (M+H)	90	8.02, 5.47
21b	OH	$CH_2CH_2NH_2$	B	527.3 (M+H)	94	7.80, 5.44
22b	$NHCH_2Bn$	$CH_2CH_2NH_2$	B	630.4 (M+H)	91	7.82, 5.70
23b	$NHCH_2Bn$	CH_2OH	B	617.2 (M+H)	93	8.05, 5.51
24b	NHBn	$CH_2CH_2NH_2$	B	616.3 (M+H)	92	8.21, 5.41

Figure S5. Two Example LCMS Spectra.



4. Biology Experimental Section

Coupled Bacterial Transcription/Translation Assay (Prokaryote)

The DNA template, pBest Luc ™ (Promega), is a plasmid containing a reporter gene for firefly luciferase fused to a strong *tac* promoter and ribosome binding site. Messenger RNA from 1 μg pBestLuc was transcribed and translated in E. coli S30 bacterial extract in the presence or absence of test compound. Compounds were tested in a black 96 well microtiter plate with an assay volume of 35 μL. Each test well contained: 5 μL test compound, 13 μL S30 premix (Promega), 4 μL 10X complete amino acid mix (1 mM each), 5 μL *E. coli* S30 extract and 8 μL of 0.125 μg/μL pBest Luc™. The transcription/translation reaction was incubated for 35 minutes at 37°C followed by detection of functional luciferase with the additon of 30 μL LucLite™ (Packard). Light output was quantitated on a Packard TopCount.

Minimum Inhibitory Concentrations (MICs, Bacteria)

The assays are carried out in 150 μL volume in duplicate in 96-well clear flat-bottom plates. The bacterial suspension from an overnight culture growth in appropriate medium is added to a solution of test compound in 2.5% DMSO in water. Final bacterial inoculum is approximately 10^2-10^3 CFU/well. The percentage growth of the bacteria in test wells relative to that observed for a control wells containing no compound is determined by measuring absorbance at 595 nm (A_{595}) after 20-24 hours at 37°C. The MIC is determined as a range of concentration where complete inhibition of growth is observed at the higher concentration and bacterial cells are viable at the lower concentration. Both ampicillin and tetracycline are used as antibiotic positive controls in each screening assay for *E. coli* (ATCC 25922) and *S. aureus* (ATCC13709).

Minimum Inhibitory Concentrations (MICs, Yeast)

The assays are carried out in 150 μL volume in duplicate in 96-well clear flat-bottom plates. The yeast suspension from an overnight culture growth in appropriate medium is added to a solution of test compound in 2.5% DMSO in water. Final yeast inoculum is approximately 10^2-10^3 CFU/well. The percentage growth of the yeast in test wells relative to that observed for a control well containing no compound is determined by measuring absorbance at 595 nm (A_{595}) after 40-48 hours (yeast) at 25°C. The MIC is determined as a range of concentration where complete inhibition of growth is observed at the higher concentration and yeast cells are viable at the lower concentration. Amphotericin B is used as a positive control for yeast MIC assays.

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Synthesis and biological evaluations of novel benzimidazoles as potential antibacterial agents

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Abstract—A series of novel benzimidazole derivatives were synthesized via parallel solution-phase chemistry. Many of these compounds were found to inhibit the growth of *Staphylococcus aureus* and *Escherichia coli*. Several analogues exhibited low micromolar minimal inhibitory concentrations (MIC) against both Gram-positive and Gram-negative bacteria of clinical relevance and could serve as leads for further optimizations for antibacterial research.

The emergence of resistance to the major classes of antibacterial agents is recognized as a serious health concern.[1–8] Particularly, the emergence of multidrug-resistant strains of Gram-positive bacterial pathogens is a problem of ever increasing significance. Organisms including methicillin-resistant *Staphylococcus aureus* (MRSA) and *Staphylococcus epidermidis* (MRSE), vancomycin-resistant enterococci (VRE), and penicillin- and cephalosporin-resistant streptococci are continually challenging scientist, physicians and patients.[5,9–14] The search for antibacterial agents with new mode of actions will always remain an important and challenging task. We have initiated a research program to discover novel antibiotics by targeting bacterial *r*RNA utilizing our unique MS-based screening technologies.[15–19] Previously, we reported the discovery of a series of novel benzimidazoles with general structure **1** that exhibit potent broad-spectrum antibacterial activities, particularly against Gram-positive bacteria (Fig. 1).[20] In this work, we report on the design and synthesis of a library of novel benzimidazoles related to **1** and the evaluation of their antibacterial activities.

To explore the SAR in the xylenyl region of these benzimidazoles and search for potentially better antibacterial agents, additional heterocycles were attached to the benzimizadole core with various linkers. The first

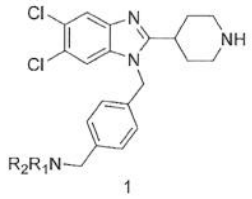

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Figure 1.

series of benzimidazole analogues contained various alkane spacers (**10–14**, Scheme 1). Since our earlier studies suggested that a nitrogen atom at the terminal site of the xylenyl moiety in **1** is important for their antibacterial activities,[20] all these new analogues bear nitrogen-containing heterocycles and their syntheses are shown in Scheme 1. 4,5-Dichloro-1,2-dianiline (**2**) reacted smoothly with *N*-Boc-isonipecotic acid (**3**) to give the corresponding amide, which cyclized upon treatment with sodium hydroxide to give benzimidazole **4**. Reaction of **4** with different diiodides furnished **5–9** in good yields. A variety of nitrogen-containing heterocycles were introduced in good yields by simple alkylation in the presence of sodium hydride or potassium carbonate. Deprotection of the Boc group furnished the target molecules **10–14** in almost quantitative yields. These benzimidazoles were first screened against *S. aureus* and *Escherichia coli*, and their minimum inhibitory concentrations (MICs) are shown in Table 1. While the simple alkyl analogues from **5–9** after removal of the Boc group exhibited no antibacterial activities, several of the heterocyclic analogues (**11i**, **13a**,**b**,**d**,**g**,**i**, **14i**) were

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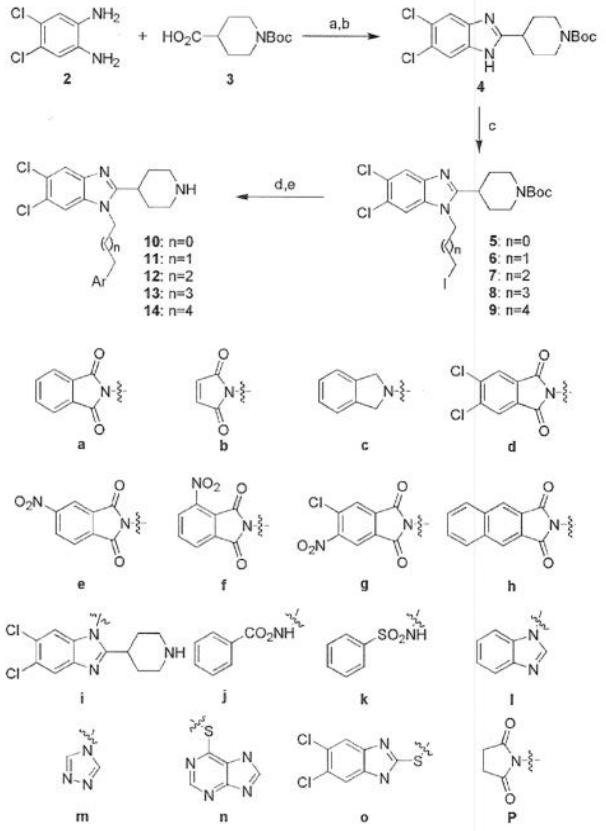
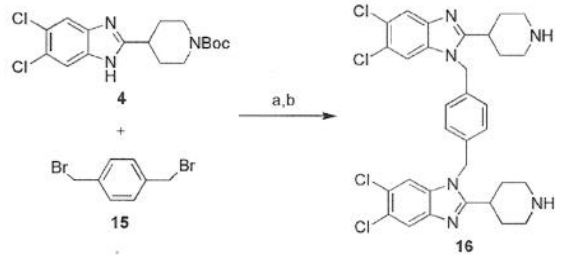


Scheme 1. Synthesis of benzimidazoles **10**–**14**. Reagents and conditions: (a) EDC (1.2 equiv), DMAP (cat.), CH_2Cl_2, 25 °C, 12 h; (b) NaOH, H_2O, 100 °C, 10 h, 65% over 2 steps; (c) $ICH_2(CH_2)_nCH_2I$ (n = 0–4, 3.0 equiv), NaH or K_2CO_3, DMF, 25 °C, 1–8 h; 69–88%; (d) ArH, NaH or K_2CO_3, 65–82%; (d) 4.0 M HCl/dioxane, CH_2Cl_2, 25 °C, 0.5 h, 90–95%.

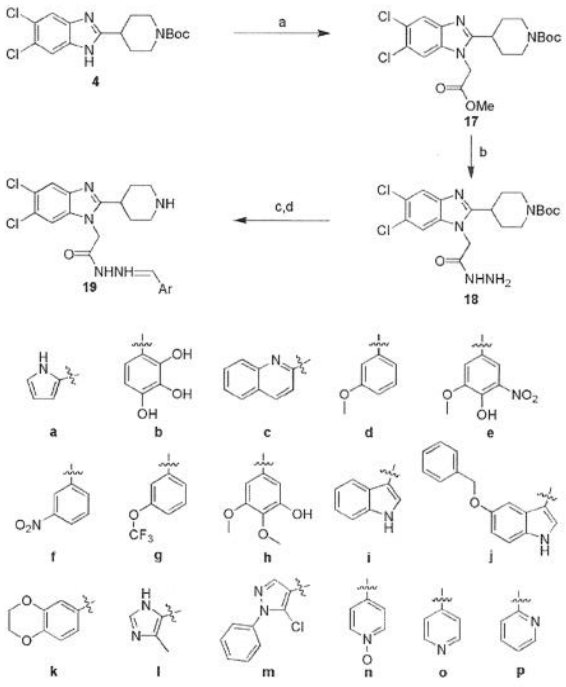
indeed found to possess good activities. Interestingly, all three dimers **11i**, **13i** and **14i** are similarly potent against both *S. aureus* and *E. coli*. Encouraged by these results, we then prepared the corresponding dimer **16**, which has the xylenyl group as the spacer. The synthesis of **16** was accomplished by first reacting **4** with 0.5 equivalents of α,α-dibromo-*p*-xylene (**15**), followed by deprotection of the Boc group using hydrogen chloride (Scheme 2). As expected, **16** exhibited low μM MICs against both *S. aureus* and *E. coli* (Table 1).

Encouraged by the antibacterial activities of these heterocyclic and dimeric benzimidazoles, we decided to further explore this heterocyclic region of these benzimidazole derivatives by synthesizing a larger library of analogues for quick screening. We thus focused on the chemistry that would be compatible with combinatorial synthesis, so that analogues could be quickly and cleanly generated for biological evaluations. A series of acylhydrazone containing various aryl or heterocyclic moieties were designed and synthesized (Scheme 3). Acylhydrazide **18** was synthesized as a key intermediate for the combinatorial generation of benzimidazoles. Since the acyl hydrazide could serve as both a hydrogen donor and acceptor to potentially add additional contacts with the target, analogues based on **18** could be potentially more potent than the parent benzimidazoles.

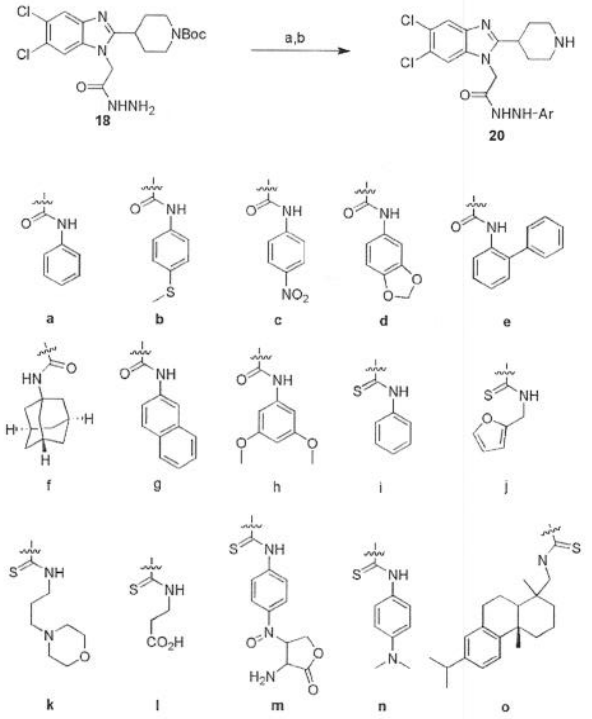
Acyl hydrazide **18** was easily prepared in gram quantity in excellent overall yield from **4** by alkylation with methyl α-bromoacetate followed by a nucleophilic displacement of the methoxy group with hydrazine. Many derivatives could then be easily synthesized in high yields from **18** without the need of vigorous purification. The first series of analogues with the general structure **19** were prepared by simply reacting **18** with different aldehydes, followed by the removal of the Boc protecting group with hydrogen chloride. All the benzimidazole analogues obtained this way have more than 95% purity based on LC/MS analysis and were thus used directly for antibacterial assays. Gratifyingly, most of these analogues (**19a–m**) inhibited *S. aureus* growth with MICs in the low μM range (Table 1). In particular, **19j** and **19m** showed 3–6 and 6–12 μM MICs against *S. aureus* respectively. In contrast to most of the active analogues found in the first library (**11i**, **13a**,**i**, **14i**, **16**) that were effective against both *S. aureus* and *E. coli*,



Scheme 2. Synthesis of benzimidazole dimer **16**. Reagents and conditions: (a) α,α-dibromo-*p*-xylene (0.5 equiv), NaH, DMF, 0 °C, 2 h, 56%; (b) 4.0 M HCl/dioxane, 25 °C, 2 h, 98%.



Scheme 3. Synthesis of benzimidazoles **18a**–**p**. Reagents and conditions: (a) NaH (3.0 equiv), $BrCH_2CO_2Me$ (1.2 equiv), DMF, 25 °C, 0.5 h, 92%; (b) H_2NNH_2 (5.0 equiv), DMF, 25 °C, 2.0 h, 98%; (c) ArCHO (1.02 equiv), CH_2Cl_2, *p*TsOH (cat.) 25 °C, 0.5 h, >95%; (d) 4.0 M HCl/dioxane, CH_2Cl_2, 25 °C, 0.5 h, >95%.



Scheme 4. Synthesis of benzimidazoles **20a–o**. Reagents and conditions: (a) RNCO or RNCS (1.05 equiv), CH_2CH_2, 25 °C, 0.5 h, >95%; (b) 4.0 M HCl/dioxane, CH_2Cl_2, 25 °C, 0.5 h, >95%.

Table 1. Inhibitory effects of benzimidazoles on *S. aureus* and *E. coli* growth[20,21]

Compd	*S. aureus* MIC (μM)	*E. coli* MIC (μM)	Compd	*S. aureus* MIC (μM)	*E. coli* MIC (μM)
10a	>100	>100	**19e**	25–50	>100
11a	>100	>100	**19f**	25–50	>100
11i	25–50	25–50	**19g**	25–50	>100
12a	>100	>100	**19h**	25–50	>100
12n	>100	>100	**19i**	25–50	>100
12o	>100	>100	**19j**	3–6	>100
13a	12–50	25–50	**19k**	50–100	>100
13b	6–12	>100	**19l**	50–100	>100
13c	50–100	25–50	**19m**	6–12	>100
13d	12–25	50–100	**19n**	>100	>100
13e	>100	50–100	**19o**	>100	>100
13f	>100	>100	**19p**	>100	>100
13g	25–50	50–100	**20a**	50–100	>100
13h	>100	>100	**20b**	12–25	25–50
13i	12–25	12–25	**20c**	25–50	>100
13j	>100	>100	**20d**	25–50	50–100
13k	>100	>100	**20e**	12–25	25–50
13l	50–100	50–100	**20f**	6–12	12–25
13m	>100	>100	**20g**	6–12	12–25
13p	>100	>100	**20h**	6–12	25–50
14a	>100	>100	**20i**	25–50	50–100
14i	6–12	12–25	**20j**	50–100	>100
16	3–6	6–12	**20k**	>100	>100
19a	12–25	>100	**20l**	>100	>100
19b	12–25	>100	**20m**	>100	>100
19c	25–50	>100	**20n**	50–100	>100
19d	25–50	>100	**20o**	25–50	25–50
19e	25–50	>100	Paromomycin	1–3	3–6

none of analogues in this series had any activity for *E. coli*. These data suggested that the spacer attached to the benzimidazole nitrogen plays an important role in determing the antibacterial activities of these analogues. All three pyridine analogues (**19n–p**) had no effect against these bacteria, which were possibly due to the presence of the basic pyridine nitrogen.

Next, a variety of isocyanides and isothiocyanides were then allowed to react with acyl hydrazide **18**, and the corresponding ureas and thioureas were obtained in excellent yields and purity (Scheme 4). The resulted *N*-Boc protected intermediates were directly treated with hydrogen chloride to give the corresponding products of general structure **20** in almost quantitative yields and more than 95% purity. In the urea series, a variety of functional moieties with different sizes were tolerated and all these analogues (**20b–h**) except **20a** showed good antibacterial activities. Interestingly, similar to the analogues with alkyl spacers, many analogues in this series again exhibited good activities against both *S. aureus* and *E. coli*. However, among the thioureas analogues, only **20i** and **20o** showed moderate activities (Table 1).

Similar to the xylenylamine analogues (**1**), most of these compounds did not show appreciable inhibitory activities in the transcription/translation assay (selected data shown in Table 2), suggesting that the antibacterial activities of these compounds are most likely not due to the inhibition of the transcription/translation machinery.

Table 2. Minimal Inhibitory Concentrations (MIC) of selected benzimidazoles against bacteria and their inhibitory concentrations (IC_{50}) in the Transcriptions/Translation (T/T) assay[a,21]

Compd	MIC (μM, Gram+)				MIC (μM, Gram−)				IC_{50} (μM)
	SA1	EH2	SP4	SP6	EC2	PV8	KP1	PA2	T/T
13a	6–12	25–50	25–50	12–25	25–50	25–50	25–50	50–100	25
13b	6–12	25–50	25–50	6–12	>100	>100	50–100	>100	>100
13i	3–7	0.75–1.5	25–50	6–12	12–26	25–50	25–50	6–12	35
14i	6–12	1–3	3–6	6–12	12–25	NT	6–12	12–25	>100
16	3–6	1–3	3–6	6–12	6–12	NT	6–12	12–25	>100
19j	3–6	3–6	6–12	12–25	>100	50–100	6–12	50–100	>100
19m	6–12	1–3	6–12	12–25	>100	25–50	25–50	50–100	>100
20f	6–12	3–6	6–12	12–25	12–25	12–25	12–25	25–50	>100
20g	6–12	3–6	6–12	12–25	12–25	25–50	6–12	25–50	>100
20h	6–12	1–3	6–12	12–25	25–50	>100	12–25	>100	>100

[a] SA1: *S. aureus* 13709; EF2: *E. hirae* 29212; SP4: *S. pyogenes* 49399; SP6: *S. pneumoniae* 6303; EC2: *E. coli* 25922; PV8: *P. vulgaris* 8427; KP1: *K. pneumoniae* 13383; PA2: *P. aeruginosa* 25416; NT: Not tested.

To further evaluate the potential of these benzimidazole derivatives, the active compounds were screened against a panel of clinically relevant bacteria, and most of these compounds were found to be active against these bacteria (Table 2). In particular, **13i**, **14i**, **16**, **20f**,**g** exhibited low μM broad-spectrum activities. The promising activities and easy access of these benzimidazole derivatives render them as very attractive antibacterial leads. Further optimizations and detailed SAR studies are the subject of future studies and shall be reported in due course.

Acknowledgements

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2-Piperidin-4-yl-benzimidazoles with Broad Spectrum Antibacterial Activities

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Abstract—A series of 2-piperidin-4-yl-benzimidazoles were synthesized and evaluated for antibacterial activities. Certain compounds inhibit bacterial growth with low micromolar minimal inhibitory concentration (MIC). These benzimidazoles are effective against both Gram-positive and Gram-negative bacteria of clinical importance, particularly entercococci, and represent a new class of potential antibacterial agents.

Almost all the major classes of antibiotics have encountered resistance in clinical applications.[1–5] The emergence of bacterial resistance to β-lactam antibiotics, macrolides, quinolones, and vancomycin is becoming a major worldwide health problem.[4–8] In particular, antibiotic resistance among Gram-positive bacteria (staphylococci, enterococci, and streptococci) is becoming increasingly serious.[9–13] Entercococci, which are frequently resistant to most antibiotics including penicillin, cephalosporin and aminoglycosides, are often treated with either a combination of two antibiotics or vancomycin. However, with the recent increased use of vancomycin in methicillin-resistance *Staphylococcus aureus* (MRSA) infections and colitis due to *Clostridium difficile*, multiple resistant *Entercoccus faecium* has been spreading.[14] As such, the last resort for anti-infective diseases, the Vancomycin family of antibiotics, has now been gravely challenged in recent years due to the emergence of Vancomycin resistance in clinical practice.[11,15]

In order to overcome these emerging resistance problems, there is an urgent need to discover novel antibacterial agents in structural classes distinct from existing antibiotics. In an effort to identify such compounds, we continuously screen our in-house libraries for active leads. Herein, we report on the initial structure–activity relationship (SAR) studies of a series of benzimidazoles that have been discovered to possess broad-spectrum antibacterial activities.

The synthesis of this class of benzimidazoles is shown in Scheme 1. Treatment of commercially available 4,5-dichloro-1,2-phenylenediamine (**1**) and *N*-Boc-isonipecotic acid (**2**) with EDC in the presence of catalytic amount of DMAP led to the formation of the corresponding amide. The crude mixture was then refluxed in aqueous sodium hydroxide solution to give cyclized intermediate **3**, which was reacted with various alkyl, benzyl and aryl halides to give **4b–4i**. Treatment of compound **4g** with various amines or nitrogen-containing heterocylces provided **6a–r**. Deprotection of the Boc group with anhydrous hydrogen chloride (HCl, 4.0 M) in dioxane at room temperature for 30 min formed benzimidazoles **7a–r**. In a similar manner, **3**, **4b–4i** were treated with hydrogen chloride to give benzimidazoles **5a–i**.

In the initial assays, all the benzimidazoles were tested for activity against *S. aureus* and *Eschericia coli*.[16] Their minimal inhibitory concentrations (MIC) are listed in Table 1. The preliminary data showed that all the amine analogues (**7a–r**) exhibited strong ability to inhibit *S. aureus* with most of the MICs in the low micromolar range. Various nitrogen substitutions are accepted including straight alkyl chain amines and diamines (**7a–g**), polyamine (**7h**), cyclic diamines (**7i**, **7j**, **7n–p**), heterocyclics (**7k–m**, **7q**, **7r**). These benzimidazoles are also effective

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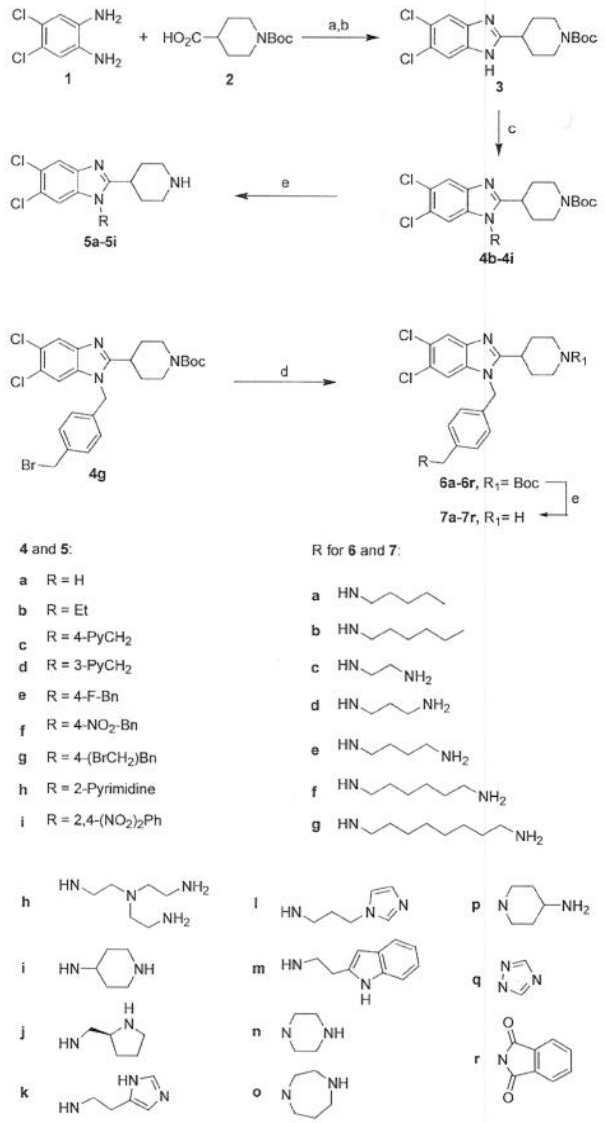

Scheme 1. Synthesis of benzimidazoles: (a) EDC, DMAP; (b) NaOH, H_2O; (c) RX, NaH or K_2CO_3; (d) for amines (RH), DMF 25 °C, 2 h; for heterocycles (RH), K_2CO_3 DMF, 25 °C, 2 h; (e) 4.0 M HCl/dioxane, 0 °C, 0.5 h.

in inhibiting *E. coli* growth, albeit with somewhat reduced potency. Nevertheless, some compounds possess MICs approaching low micromolar concentrations. In comparison, these benzimidazoles are two or more folds more effective against *S. aureus* than *E. coli*. In contrast, compound **5a**, which has no substitution at N1 position, and compounds **5b–i**, which have various alkyl, benzyl or aryl substitutions at N1 positions did not exhibit any appreciable ability to inhibit bacterial growth. This suggests the crucial role of a basic nitrogen distinct from the benzimidazole core.

All benzimidazoles were also screened for their ability to inhibit bacterial translation and transcription using a coupled assay.[16] Several compounds (**7c**, **7d**, **7h**, **7k**) were found to posses low micromolar IC_{50}s. Since these compounds have similar inhibitory activities against *S. aureus* and *E. coli*, their antibacterial activities could

Table 1. Inhibitory effects of benzimidazoles on *S. aureus* and *E. coli* growth and bacterial transcription/translation[17]

Compd	*S. aureus* MIC (μM)	*E. coli* MIC (μM)	T/T IC_{50} MIC (μM)
5a	>100	>100	>100
5b	>100	>100	>100
5c	50–100	50–100	>100
5d	50–100	50–100	>100
5e	>100	>100	>100
5f	50–100	>100	>100
5g	>100	>100	>100
7a	6–12	12–25	>100
7b	3–6	6–12	>100
7c	6–12	12–25	12
7d	12–25	50–100	20
7e	6–12	25–50	50
7f	6–12	12–25	>100
7g	6–12	6–12	>100
7h	3–6	12–25	10
7i	6–12	12–25	>100
7j	12–25	12–25	>100
7k	6–12	12–25	25
7l	12–25	12–25	>100
7m	12–25	12–25	>100
7n	6–12	12–25	>100
7o	6–12	12–25	>100
7p	6–12	50–100	60
7q	12–25	12–25	>100
7r	6–12	6–12	>100
Ciprofloxacin	0.75–1.56	0.75–1.56	>100
Paromomycin	1–3	3–6	0.56

be partially due to the inhibition of bacterial transcription and/or translation. However, most of the IC_{50} values are much higher than the corresponding MICs for *S. aureus* and *E. coli*, it is unlikely that the antibacterial activities for most of these compounds are due to inhibition of either bacterial transcription or translation.

To test effectiveness of these benzimidazoles against other bacteria, the active compounds from the preliminary screening were screened against additional four strains of Gram-positive and four strains of Gram-negative bacteria, and the results are shown in Table 2. Again, these compounds exhibited higher potencies against Gram-positive bacteria (*S. aureus* 13709, *Enterococcus hirae* 29212, *Streptococcus pyogenes* 49399, and *Streptococcus pneumoniae* 6303) as compared to Gram-negative bacteria (*E. coli* 25922, *Proteus vulgaris* 8427, *Klebsiella pneumoniae* 13383, *Pseudomonas aeruginosa* 25416). Several benzimidazoles, in particular **7b**, **7f** and **7g**, showed interesting activities against *E. hirae*. These compounds were screened against seven additional clinically important *Enterococcus* strains, and the results are shown in Table 3. In addition to their original activities, several compounds (**7a**, **7b** and **7r**) displayed strong inhibitory activities against all eight *Enterococcus* strains. It appears that relatively hydrophobic terminal moieties could enhance the antibacterial activities against *Enterococcus*. Further studies are necessary to fully understand their potent antibacterial activities. To study the selectivity for inhibition of bacterial growth, these compounds were also screened against yeast cell line *Candida albicans* 10231 (Table 2). Certain compounds were significantly less inhibitory to yeast growth

Table 2. Minimal inhibitory concentrations (MIC, μM) of benzimidazoles against bacteria and yeast[a]

Compd	Gram+				Gram−				Yeast
	SA1	EH2	SP4	SP6	EC2	PV8	KP1	PA2	CA1
7a	6–12	1–3	3–6	12–25	12–26	25–50	6–12	25–50	50–100
7b	3–6	1–3	3–6	6–12	6–12	12–25	6–12	25–50	25–50
7c	6–12	3–6	6–12	12–25	12–25	25–50	12–25	12–25	>100
7d	12–25	6–12	6–12	25–50	50–100	NT	25–50	25–50	>100
7e	6–12	3–6	6–12	25–50	25–50	NT	25–50	25–50	>100
7f	6–12	1–3	3–6	12–25	12–25	25–50	12–25	25–50	>100
7g	6–12	1–3	3–6	6–12	6–12	12–25	6–12	25–50	>100
7h	3–6	3–6	3–6	6–12	12–25	25–50	12–25	12–25	>100
7i	6–12	3–6	6–12	12–25	12–25	25–50	12–25	12–25	50–100
7k	6–12	3–6	6–12	12–25	12–25	25–50	12–25	25–50	>100
7n	6–12	3–6	6–12	12–25	12–25	12–25	6–12	12–25	50–100
7o	6–12	3–6	6–12	12–25	12–25	25–50	12–25	25–50	>100
7p	6–12	3–6	6–12	25–50	50–100	25–50	25–50	12–25	50–100
7r	6–12	3–6	12–25	6–12	6–12	25–50	25–50	12–25	50–100

NT, not tested.
[a]SA1, *S. aureus* 13709; EH2, *E. hirae* 29212; SP4, *S. pyogenes* 49399; SP6, *S. pneumoniae* 6303; EC2, *E. coli* 25922; PV8, *P. vulgaris* 8427; KP1, *K. pneumoniae* 13383; PA2, *P. aeruginosa* 25416; CA1, *C. albicans* 10231.

Table 3. Minimal inhibitory concentrations (MIC, μM) of benzimidazoles against *Enterococcus*

Compd	*E. faecalis* ATCC 11823	*E. faecalis* ATCC 23241	*E. faecalis* ATCC 4200	*E. faecalis* ATCC 49757	*E. faecalis* ATCC 828	*E. faecium* ATCC 6569	*E. faecium* ATCC 882	*E. faecium* ATCC 29212
7a	3–6	6–12	6–12	6–12	6–12	3–6	6–12	1–3
7b	3–6	3–6	3–6	3–6	3–6	3–6	3–6	1–3
7c	6–12	12–25	12–25	12–25	12–25	6–12	12–25	3–6
7d	12–25	25–50	25–50	12–25	12–25	12–25	25–50	6–12
7e	12–25	12–25	12–25	12–25	12–25	12–25	12–25	3–6
7g	6–12	NT	25–50	50–100	50–100	12–25	NT	1–3
7l	12–25	12–25	12–25	12–25	12–25	12–25	12–25	3–6
7l	6–12	12–25	12–25	12–25	6–12	6–12	12–25	3–6
7n	12–25	6–12	12–25	12–25	6–12	6–12	6–12	3–6
7o	12–25	12–25	12–25	6–12	12–25	6–12	12–25	3–6
7p	12–25	12–25	12–25	12–25	12–25	6–12	12–25	3–6
7r	3–6	6–12	6–12	6–12	6–12	3–6	6–12	3–6

NT, not tested.

as compared to bacterial growth, suggesting a selectivity index for inhibition of bacterial growth.

In summary, we have discovered a novel series of benzimidazoles that exhibit a broad spectrum of antibacterial activities. These benzimidazoles are particularly effective against Gram-positive bacteria, including clinically relevant strains of *Enterococcus*. The simplicity of their structures and their high potencies against different types of bacteria render these benzimidazoles interesting leads for further investigation. The preliminary SAR has pointed to a high degree of tolerance for structure modifications at the N1 of the benzimidazole core. The effect of extensive modification around this core will be the subject of further optimization studies, which shall be reported in due course.

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17. The assays were carried out in 150 mL volume in duplicate in 96-well clear flat-bottom plates. The growth bacterial or yeast suspension from an overnight culture in appropriate medium was added to a solution of test compound in 2.5% DMSO in water. Final bacterial or yeast inoculum is approximately 102–103 CFU/well. The percentage growth of the bacteria or yeast in test wells relative to that observed for a control well containing no compound was determined by measuring absorbance at 595 nm (A595) after 20–24 h at 37 °C (bacteria) or 40–48 h (yeast) at 25 °C. The MIC was determined as a range of concentration where complete inhibition of growth is observed at the higher concentration and bacterial/yeast cells are viable at the lower concentration.

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Aryl urea analogs with broad-spectrum antibacterial activity

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Abstract—The preparation and evaluation of novel aryl urea analogs as broad-spectrum antibacterial agents is described. Numerous compounds showed low micromolar minimum inhibitory concentrations (MIC) against both Gram-positive and Gram-negative bacteria. Selected analogs also exhibited in vivo efficacy in a lethal murine model of bacterial septicemia.

The emergence of drug resistant pathogenic bacteria continues to be a serious health problem worldwide.[1] As a result, it has become critical to identify new structural classes of antibacterial agents to combat the growing threat of bacterial resistance.[2] During screening of new compound classes for antibacterial activity, thiourea **1** (Fig. 1) was identified as having good activity[3] (MIC 3–6 μM) against *S. aureus* in our assay.[4] However, thiourea **1** did not show any antibacterial activity in the in vitro assay (MIC > 100 μM) when tested in the presence of 4% bovine serum albumin (BSA). Since the loss of activity in the presence of serum could be attributed to the high lipophilicity of thiourea **1**, we initiated a study to identify alternate structures with reduced lipophilicity and equivalent or improved antibacterial activity. Here we report the initial SAR study around thiourea **1** resulting in analogs with good broad-spectrum antibacterial activities as well as in vivo efficacy in a lethal murine model of bacterial septicemia.

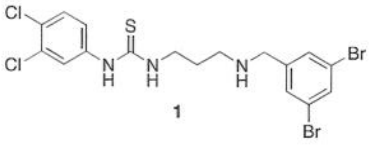
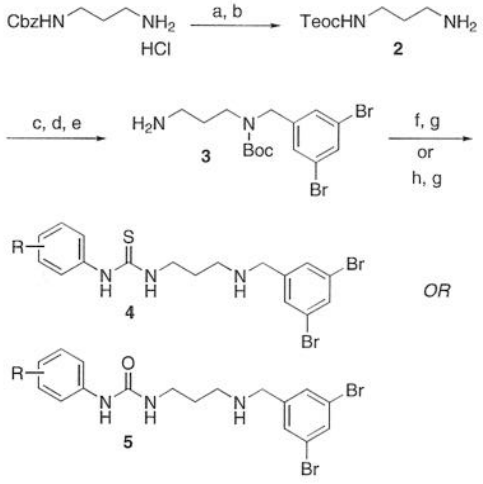


Figure 1.

We first chose to modify the aryl ring of the thiourea moiety without altering the 3,5-dibromo substitution pattern on the *N*-benzyl substituent. Commercially available mono-Cbz protected 1,3-diaminopropane was reacted with Teoc-OSu, followed by removal of the Cbz group by catalytic hydrogenation to provide mono-Teoc protected 1,3-diaminopropane **2** (Scheme 1). Reductive amination of **2** with 3,5-dibromobenzaldehyde,



Scheme 1. Reagents and conditions: (a) Teoc-OSu, Et_3N, CH_2Cl_2; (b) 10% Pd/C, H_2; (c) 3,5-dibromobenzaldehyde, $NaBH_3CN$, MeOH (68%, over three steps); (d) Boc_2O, CH_2Cl_2 (95%); (e) 1 M TBAF, KF, CH_3CN, 40 °C, 10 h (75%); (f) ArNCS, CH_2Cl_2; (g) 30% TFA/CH_2Cl_2; (h) ArNCO, CH_2Cl_2.

Keywords: Aryl urea; Antibacterial; Broad spectrum.
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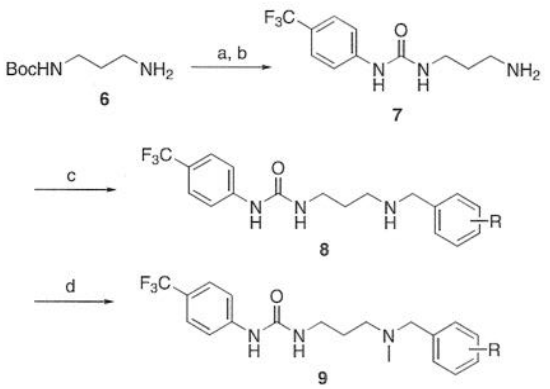
protection of the secondary amine with Boc_2O and deprotection of the Teoc group using TBAF/KF provided amine **3**. Subsequent reaction of **3** with commercially available arylisothiocyanates or arylisocyanates followed by deprotection of the Boc group provided the corresponding thiourea **4** or urea **5** analogs, respectively, in good overall yield (40–55% after reversed phase HPLC purification). All final thiourea **4** and urea **5** analogs were tested for antibacterial activity as their acetate salts.

In the first SAR set, ~30 thiourea/urea analogs with electron donating and electron withdrawing groups at the 2-, 3-, and 4-positions of the aryl ring were prepared and evaluated. The objective was to identify substitutions that would reduce hydrophobicity while maintaining activity. Biological screening revealed that substitution at the 2-position was not tolerated and led to a substantial decrease in antibacterial activity (Table 1). Substitution at the 3- and 4-positions was tolerated and optimal activity was seen with small non-polar groups such as chloro and trifluoromethyl. From this series urea **5d** with a 4-CF_3 group exhibited activity similar to the lead thiourea **1**. Urea analog **5d** also had a lower Clog*P* value[5] (4.89) as compared to thiourea **1** (6.23). As a result we chose urea **5d** as the new lead structure for further optimization.

In the second analog set, we explored the SAR of the benzylamino substituent. Commercially available *N*-Boc-1,3-diaminopropane was reacted with 4-(trifluoromethyl)phenyl isocyanate followed by deprotection of the Boc group to provide urea **7** (Scheme 2). Reductive aminations with ~40 aromatic aldehydes provided ureas **8** (40–55% after reversed phase HPLC purification). All the final compounds were tested for antibacterial activity as their acetate salts.



Scheme 2. Reagents: (a) 4-CF_3-phenyl isocyanate, CH_2Cl_2; (b) 30% TFA, CH_2Cl_2 (90% over two steps); (c) ArCHO, TMOF, $NaBH_3CN$, cat. AcOH, MeOH; (d) 40% HCHO, MeOH, cat. AcOH, $NaBH_3CN$.

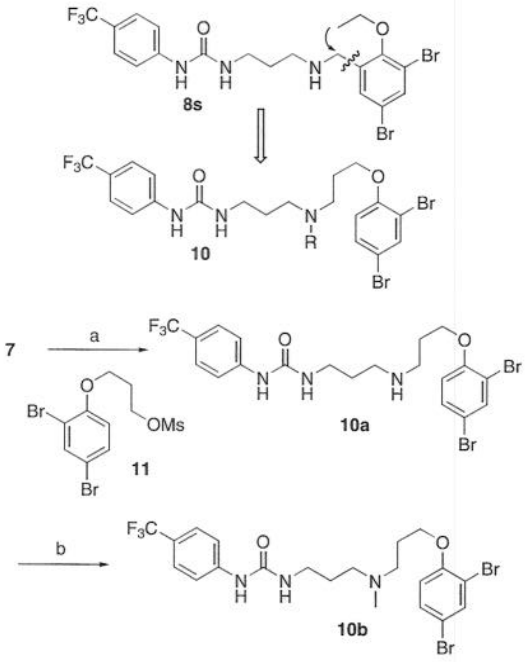
Substitution at the 4-position of the benzylamino group with a variety of hydrophobic substituents such as *t*-butyl, phenyl, phenoxy, and OCF_3 provided analogs that showed good activity against *S. aureus* (Table 2, entries 4–9). We were also surprised that a 4-dimethylamino group also displayed some antibacterial activity suggesting that it may be possible to substitute the 4-position with other hydrophilic groups (entry 10). The best substitution pattern for the halogenated benzylamino analogs was either 3,5-dibromo (**8l**) or 3,4-dichloro (**8q**). 3,5-Dichloro-substitution resulted in a slight loss of activity while 3,5-difluoro, 3,5-ditrifluoromethyl, and 3,5-dimethoxy analogs showed reduced

Table 1. In vitro antibacterial activity of thiourea/urea analogs

Entry	Compd	R	MIC (μM)[4] *S. aureus*
1	**1**	3,4-DiCl	3–6
2	**4a**	2-F	25–50
3	**4b**	2-Cl	12–25
4	**4c**	2-OMe	>100
5	**4d**	3-F	12–25
6	**4e**	3-Cl	6–12
7	**4f**	3-Br	6–12
8	**4g**	3-CF_3	6–12
9	**4h**	3-OMe	25–50
10	**4i**	4-F	12–25
11	**4j**	4-Cl	6–12
12	**4k**	4-Br	12–25
13	**4l**	4-I	12–25
14	**4m**	4-CF_3	6–12
15	**4n**	4-NO_2	12–25
16	**4o**	4-CH_3	12–25
17	**4p**	4-CN	25–50
18	**4q**	4-OMe	25–50
19	**5a**	3-CF_3	6–12
20	**5b**	3,4-DiCl	3–6
21	**5c**	4-Cl	6–12
22	**5d**	4-CF_3	3–6
23	**5e**	4-OCF_3	6–12
24	Linezolid	—	3–6

Table 2. In vitro antibacterial activity of benzylamino group modified analogs

Entry	Compd	R	MIC (μM)[4] *S. aureus*
1	**8a**	H	50–100
2	**8b**	2-OCF_3	12–25
3	**8c**	3-Ph	6–12
4	**8d**	4-OCF_3	3–6
5	**8e**	4-Ph	3–6
6	**8f**	4-(4′-OMe)-Ph	3–6
7	**8g**	4-(2′-OMe)-Ph	6–12
8	**8h**	4-OPh	3–6
9	**8i**	4-*t*-Bu	3–6
10	**8j**	4-NMe_2	12–25
11	**8k**	4-NHCOMe	>100
12	**5d**	3,5-DiBr	3–6
13	**8m**	3,5-DiCl	6–12
14	**8n**	3,5-DiF	50–100
15	**8o**	3,5-DiCF_3	50–100
16	**8p**	3,5-DiOMe	50–100
17	**8q**	3,4-DiCl	3–6
18	**8r**	2-OH-3,5-DiBr	>100
19	**8s**	2-OEt-3,5-DiBr	3–6
20	**8t**	2-OEt-5-Br	6–12
21	**9**	3,5-DiBr	>100
22	**10a**	—	3–6
23	**10b**	—	6–12
24	Linezolid	—	3–6



Scheme 3. Reagents and conditions: (a) **11**, $CaCO_3$, DMF, 40 °C, 48 h; (b) 40% HCHO, MeOH cat. AcOH, $NaBH_3CN$.

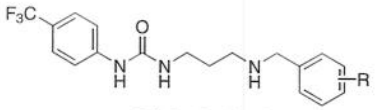
antibacterial activity (entries 12–17). Replacement of the aryl ring with halogenated heteroaromatic rings was also not well tolerated (data not shown). A few urea analogs with substituted and unsubstituted phenolic groups were also prepared and evaluated (entries 18–20). Our intention was to incorporate groups that could potentially provide us with a handle to introduce alkyl chains with polar substituents. In this series, urea **8s** with a 2-ethoxy substituent showed activity comparable to the lead urea **5d**. Interestingly, analogs **10a–b** with extended tethers (Scheme 3) were prepared and found to be as active as the 2-ethoxy substituted analog **8s** (entries 22–23). An interesting observation is that replacement of the secondary amino group in the tether with a tertiary amine was permitted in case of urea **10b** but not for urea **9** (entry 21). It would appear that the methyl group in **9** might alter the conformation such that the molecule is not able to bind its target. In contrast, analog **10b** may be flexible enough to adopt the bioactive conformation despite the methyl group on the tether amine.

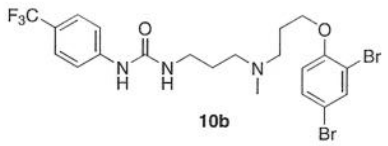
A number of analogs were then evaluated against a broader panel of Gram-positive and Gram-negative bacteria (Table 3). For the most part, urea analogs showed low micromolar activity against *S. aureus*, *S. pyogenes*, and *E. faecalis*. However, only ureas **8i** and **8s** showed good activity against *E. faecium*. Most of the urea analogs showed slightly reduced activities against *E. coli* but good activity against *K. pneumoniae*. All compounds showed weak activity against *P. vulgaris* and no activity against *P. aeruginosa*.

Selected urea analogs were also evaluated for activity in the presence of 4% BSA. Most of the analogs tested showed reduced activity in the presence of serum (Table 3). This was not completely surprising considering the high Clog *P* values for these compounds (Table 3). The greatest reduction in antibacterial activity in the presence of serum was observed for the halogenated analogs **5d**, **8s** and **10b**. Ureas **8e** and **8h** gave the best activity in the presence of serum (4-fold reduction).

Analysis of the in vitro data suggests that the urea analogs are binding in a very lipophilic binding pocket as increasing hydrophobicity improves the activity. The antibacterial activity of the urea analogs is also reduced substantially in the presence of 4% bovine serum albumin. It is conceivable that the reduced activity is likely the result of high serum protein binding of this compound class. These observations are consistent with the previous SAR studies carried out on other urea

Table 3. Broad-spectrum antibacterial activity of urea analogs





Bacteria	MIC (μM)						
	5d 3,5DiBr	**8s** 2-OEt-3,5-DiBr	**8e** 4-Ph	**8h** 4-OPh	**8i** 4-*t*-Bu	**10b**	Linezolid
S. aureus ATCC 13709	3–6	3–6	3–6	3–6	3–6	3–6	3–6
S. pyogenes ATCC 49399	6–12	3–6	3–6	3–6	3–6	3–6	1.5–3
E. faecalis ATCC 29212	6–12	6–12	>100	3–6	6–12	3–6	3–6
E. faecium ATCC 6569	>100	6–12	>100	>100	3–6	>100	>100
E. coli ATCC 25922	12–25	12–25	6–12	6–12	6–12	12–25	>100
K. pneumoniae ATCC 13383	6–12	3–6	6–12	6–12	3–6	3–6	>100
P. vulgaris ATCC 8427	12–25	12–25	12–25	12–25	12–25	12–25	12–25
P. aeruginosa ATCC 25416	>100	>100	>100	>100	>100	>100	>100
S. aureus ATCC 13709 (+4% BSA)	25–50	25–50	12–25	12–25	50–100	25–50	3–6
MTT HUH-7—CC_{50} 48 h (μM)	NT	50–100	25–50	12.5–25	NT	>100	NT
Clog *P*	4.89	5.1	4.91	4.77	4.94	5.3	0.58

Table 4. In vivo antibacterial activity of urea analogs[13]

Entry	Compd	Dose (mg/kg)	Mice alive/total
1	No drug	—	0/10
2	**10b**	75 × 2	4/10
3	**10b**	37.5 × 2	7/10
4	**10b**	18.8 × 2	6/10
5	**10b**	9.4 × 2	6/10
6	**10b**	4.7 × 2	4/10
7	**10b**	2.3 × 2	3/10
8	**8e**	37.5 × 2	3/10
9	**8e**	18.8 × 2	3/10
10	**8e**	9.4 × 2	4/10
11	**8e**	4.7 × 2	2/10
12	**8e**	2.3 × 2	0/10
13	Vancomycin	1 × 2	10/10

based antibacterial compounds.[6–9] While increased serum binding is not necessarily an undesirable property for antibiotic compounds, it indicates that higher doses of compound may be required to elicit a therapeutic response in vivo[10,11] This may narrow the therapeutic window and also raise the aqueous solubility threshold for poorly soluble drugs. During the course of this study we were able to prepare analogs with reduced lipophilicity and improved aqueous solubility relative to thiourea **1**.[12] However, there is still scope for reducing the lipophilicity and serum protein binding as well as increasing the potency of this compound class.

Lastly, to assess the in vivo efficacy of this class, analogs **8e** and **10b** were advanced for testing in a lethal murine model of bacterial infection (Table 4).[13] During the in vivo evaluation, urea **10b** showed efficacy in the murine model with 6/10 mice surviving in the 9.4 mg/kg (dosed 1 and 3 h postinfection) dose group and 7/10 mice surviving in the 37.5 mg/kg (dosed 1 and 3 h postinfection) dose group. Urea **10b**, however, was unable to rescue all mice from infection at the higher doses that were evaluated. Presumably the toxicity of the drug becomes an issue at higher drug concentrations.[13] Both urea analogs **8e** and **10b** were not as potent as the vancomycin positive control, which rescued all the animals at a dose of 1 mg/kg (dosed 1 and 3 h postinfection).

In conclusion, we have described the preparation and evaluation of novel aryl urea analogs as broad-spectrum antibacterial agents. Numerous compounds showed low micromolar activity against both Gram-positive and Gram-negative bacteria. Selected analogs also exhibited in vivo efficacy in a lethal murine model (*S. aureus*) of bacterial infection.

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3. Thiourea **1** showed activity comparable to Linezolid (3–6 μM), the positive control for our in vitro antibacterial assays.
4. The MIC assays were carried out in a 150 μL volume in duplicate in 96-well clear flat-bottom plates. The bacterial suspension from an overnight culture growth in the appropriate medium was added to a solution of test compound in 0.5% DMSO in water. Final bacterial inoculum was approximate 10^3–10^4 CFU/well. The percentage growth of the bacteria in the test wells relative to that observed for a control well containing no compound was determined by measuring absorbance at 595 nm (A_{595}) after 20–24 h at 37 °C. The MIC was determined as a range of concentrations where complete inhibition of growth was observed at the higher concentration and the bacterial cells were viable at the lower concentration. The bacterial strains used for the assays include *S. aureus* ATCC 13709, *S. pyogenes* ATCC 49399, *E. faecalis* ATCC 29212, *E. faecium* ATCC 6569, *E. coli* ATCC 25922, *K. pneumoniae* ATCC 13383, *P. vulgaris* ATCC 8427, *P. aeruginosa* ATCC 25416.
5. Clog *P* values were calculated using ChemDraw Ultra 7.0 software.
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12. Solubility of **8e**·lactate salt ~8 mg/mL, solubility of **10b**·HCl salt >10 mg/mL.
13. Mouse protection assay: Ten mice/dose group (ICR-CD-1 female mice 18–20 g, Charles River) were infected with a lethal dose (10^6 CFU/mouse) of *S. aureus* (ATCC 13709) suspended in 7.5% hog Gastric Mucin (IP). The infected animals were treated at 1 and 3 h postinfection with either compound **8e**·lactate salt, from 37.5 mg/kg down to 2.3 mg/kg or compound **10b**·hydrochloride salt, from 75 mg/kg down to 2.3 mg/kg (0.1 mL/mouse, IV). The positive control drug was vancomycin 1 mg/kg dosed twice at 1 and 3 h postinfection. The animals were observed for one week and mortality was calculated. Ureas **8e** and **10b** were toxic when dosed at concentrations above 37.5 and 150 mg/kg, respectively.